NEW PERSPECTIVES ON THE INTERVAL-STRENGTH RELATIONSHIP OF GUINEA PIG ATRIUM

Toward an Understanding of Mechanisms Coupling Membrane Excitation and Contraction in Heart

DISSERTATION

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

der Fakultät für Biologie
der Eberhard-Karls-Universität Tübingen

vorgelegt von

DONALD HILGEMANN
aus Postville, Iowa, USA

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To Mom

--and the hope, however small, that our science
will some day serve some real human needs.

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Preface.

It is now more than seven years since I began work for Professor Siess, helping with the english correspondence and manuscripts, as well as occasionally helping with an experiment. I am deeply indebted to Professor Siess for allowing me to specialize more in doing experiments than in writing English, an ability which I have lost to a large extent over the years in Tübingen. There came numerous times in the course of this work when it seemed like the bread-on-the-table and the guinea-pig-atrium-in-the-muscle chamber were in existential danger. For the many times that he rescued a position for me I am thankful. For his suggestions, corrections and help with manuscripts, abstracts and applications I am grateful. For his support, encouragement and discussions I express my hearty thanks and appreciation to Professor Siess.

Dr. Mensing is responsible for much of the theoretical and methodological foundations of the work presented in this dissertation. Also, without his private financial investment in the project the work would not have been possible. Whereas Dr. Mensing has been intensively interested in the problem of excitation-contraction coupling as long as I have known him, I myself have tried to avoid as much as possible getting tied up in problems other than those which were immediately posed by the experiments at hand. He advocated basically the excitation-contraction model described in chapter 8 and the necessity of simulating the model (in a more detailed way than presented here) five years ago. I am grateful for our association and discussions over the years. I thank Dr. Mensing as well for his advice and collaboration.

Dipl. Ing. R. Englert built the frequency generator and wrote the curve-fitting and -plotting program used in this dissertation, and without which the work would have been impossible. I thank him not only for this work, but for being my mathematics teacher as well. R. Englert was a real source of solace in many times of mathematical woe.

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To all members of the shop I express my thanks for their quick help when something needed to be built or repaired.

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Finally, a special thanks is expressed to Ms. B. Hirth for loaning me her typewriter for several weeks.

"...the British discriminate carefully between 'hill walking' and 'mountain climbing'. To be a mountain climber, you must coil 100 feet of nylon rope around you slantwise from shoulder to waist, and have a few pitons dangling somewhere about. You are then entitled to adopt an ever-so-faintly condescending attitude toward any hill-walkers whom you may encounter along the trail, even if you meet them where the trail is practically level. Hill-walkers, on the other hand, remain hill-walkers even when the 'walk' turns into a hands-and-knees job up a 40° slope of talus which is barely anchored to the mountain by a few wisps of grass and a clump or two of scraggly heather."

--Douglas Shepard Riggs (1963)
in The Mathematical Approach to Physiological Problems.

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1. Introduction.

The discovery that isolated heart muscle preparations can be stimulated to contract by small electrical shocks revealed directly a highly dynamic quality of cardiac muscle; the strength of contraction depends strongly on the pattern of stimulation. In 1871 Bowditch described the fundamental behavior of frog ventricle in response to electrical stimulation. 1) In contrast to skeletal muscle, the heart responds to electrical stimulation of varying strength either with a contraction or with none. Contraction strength under controlled conditions is not augmented by increasing the electrical stimulation above the level needed to just elicit contractions§. 2) When muscle preparations are paced at a regular beat interval, contraction force increases with decrease of the interval between beats. Above certain frequencies the force of contraction again decreases. 3) If electrical stimulation is stopped for a period, and then restarted, contraction force increases in a number of beats up to the steady state level ("Bowditch staircase").

At the turn of the century Woodworth (1902) extended Bowditch's work on frog ventricle to mammalian ventricular muscle of dog. The general patterns found for frog heart were confirmed. Also, several contractile phenomena in response to electrical stimulation were described for mammalian ventricle, which are not pronounced or absent in frog heart. If a train of regular impulses is broken by a somewhat longer interval, the strength of this contraction is greater than the previous contractions. Conversely, if an extra beat falls during a regular train of impulses--the beat interval being shorter than the regular beat interval--the strength of this extra beat is less than the beats at the regular interval. The subsequent few beats after the extra beat are larger than the steady state contraction strength. Woodworth thought of the effects described in terms of a "stimulating" effect of con-

§ As is well-known, this statement must be qualified. Strong electrical stimulation can indeed lead to a marked positive inotropic effect due to release of endogenous catecholamines (Blinks and Koch-Weser, 1963). Bowditch apparently did not observe this effect.

secutive contractions and a "recuperative" effect of long pauses.

From the wealth of literature which appeared on the interval-strength relationship between 1900 and the early 1960's, the most important work with regard to this dissertation is that of Kruta and coworkers (1938, 1960, 1964). Interval-strength relationships of atria of various species were studied. 1) In contrast to most other heart preparations, mammalian atrial muscle developes strong contraction force at very long beat intervals. Steady state contraction force decreases with increase of frequency from long beat intervals until a minumum is reached at 0.1 to 0.4 Hz (the "pessimum"). At higher frequencies the relationship resembles other preparations; force rises with increase of frequency, up to a limit,and then declines with still higher frequencies. Kruta emphasized that the lower portion of the atrial interval-strength relationship might prove to be important: "The middle portion of the triphasic curve seems most important because it comprises the range of frequencies occurring in the heart in the body, and most considerations of the interval-strength relationship are concerned with this sector. We cannot neglect, however, the marginal portions, which may be even more important, in searching the basic underlying phenomena.§"

2) Changes of the bath temperature have a complex effect on the relationship. Reduction of the bath temperature increases contraction strength at frequencies above the pessimum and decreases contraction strength at frequencies below the pessimum, whereby the pessimum itself is shifted to lower frequencies. Thus, the so-called "Kälteinotropie", increase of contraction force with reduction of temperature, applies only to a certain frequency range.

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§ It is useful to have terms to talk about the two main parts of the atrial steady state frequency-force relationship. The part corresponding to fall of force with increase of frequency in the low frequency range has been called the Woodworth part (Hajdu, 1969). This is a mistake,since Woodworth never worked with atrial muscle. Therefore, this part of the relationship will be called the Kruta part in this dissertation. The section of the relationship corresponding to increase of force with increase of frequency will be called the Bowditich part.

3) Kruta studied thoroughly the dependence of contraction force on the interval immediately preceding an individual beat. After a regular series of beats, the strength of test contractions is studied in dependence on the interval to the last regular beat. The ability of the heart to contract is impaired or inhibited immediately after each beat, although electrical excitability is intact just after, or even before, relaxation is completed. The curves, which describe the recovery of contractility at various intervals after a regular beat train, are called "restitution" curves. In the continuation of the restitution beyond the regular beat interval, contraction force continues to rise to a level considerably higher than the steady state contraction strength. This phenomenon, sometimes called "post-stimulatory potentiation," is more pronounced in atrial muscle than either mammalian ventricle or frog heart.

4) Kruta recognized fully the importance of the interval-strength relationship for pharmacological investigations on the heart. In order to examine the true inotropic effects of a given agent on the heart, paced muscle preparations must be used. In spontaneously-beating heart preparations chronotropic and inotropic effects cannot be separated. Finally, Kruta found it meaningful and important to examine the inotropic effects of drugs on the interval-strength relationship. Even if the processes underlying the relationships are not understood, such studies will at the least characterize an inotropic action better than just the labels, positive or negative inotropic. The effects of some agents can appear identical at a given frequency, but clear differences may be found by studying their actions on the various phenomena of the interval-strength relationship.

An in-depth review of the interval-strength relationship with several hundred citations appeared in 1963 (Koch-Weser and Blinks). This critical overview remains to date the most thorough documentation of the subject. Koch-Weser and Blinks recognized as well that the interval-strength relationship would prove to be a useful tool in coming research directed toward a better understanding of the cellular function

of the heart. Their work is characterized by a great effert to reduce as many observations as possible to the simplest common denominator. Blinks and Koch-Weser (1961) proposed that most observations on the interval-strength relationships of different species and heart muscle types could be accounted for in terms of two processes, a negative and a positive inotropic effect of activation (NIEA and PIEA). A method was developed to quantitatively analyze these effects in atrial muscle. This method has only seldomly been applied, and the basic premises made for the analysis have not been systematically reappraised. It is surprising how little attention the Blinks and Koch-Weser analysis has received, in light of the fact that their 1963 overview is one of the most-cited papers in this field.

The analysis assumes that each beat results in the production of both NIEA and PIEA, opposed effects which will exert an influence on the strength of subsequent beats. Only beats at extremely long intervals are not subject to the influence of NIEA and PIEA, "rested state contractions." At very long intervals the NIEA and PIEA will have completely decayed between beats. In mammalian atrium contraction strength declines in the Kruta part of the frequency-force relationship because the NIEA dominates over the PIEA. In the Bowditich part of the relationship the PIEA comes to dominate over the NIEA. The effects of activation are additive in nature, so that under any conditions the contraction strength is equal to; Rested State - NIEA + PIEA.

The PIEA can be quantitatively analyzed by following the exponential decline of force for a step reduction of frequency from a relatively high rate to a low rate. After subtracting the base-line force of such a transient and plotting the log values of force against time, the amount of PIEA decaying and the rate of its decay can be quite accurately determined. NIEA is more tedious to analyze. In contrast to the PIEA, the accumulation and decay of NIEA in responses to step frequency change take place in a certain number of beats and not with any given time constant. None-the-less, the NIEA can be analyzed by exploiting the fact that a rested state contraction in atrium produces a very large negative effect.

The decay of this effect can be followed by studying the dependence of the strength of the first beat after a rested state contraction on the interval between the rested state contraction and the test contraction. The procedure is basically the same as that used by Kruta to follow the restitution of contraction between beats at higher frequencies. The NIEA decay curve is complex, showing a fast and slow part. The rate of the fast part of the curve is similar to the rate of restitution as depicted by the Kruta method. The slow part of the NIEA decay curve may take several minutes to reach nearly complete decay.

The effects of heart glycosides, adrenergic stimulation and cholinergic stimulation were analyzed in this context. In atrium, heart glycosides increase the rested state contractions in low concentrations with little effect on the PIEA and NIEA. In higher concentrations the NIEA decay is somewhat accelerated (Koch-Weser et al., 1962; 1964). Norepinephrine enhances the PIEA produced per beat in concentrations which have little effect on the rested state contractions. Accordingly, the positive inotropic effect of low concentrations of norepinephrine is greater at intermediate and high frequencies than at the lowest frequencies (Koch-Weser et al., 1964). The effects of carbachol are opposite to those of norepinephrine; the rested state contractions are left unaffected by concentrations of carbachol which decrease the PIEA produced per beat. Accordingly, carbachol has little effect on the rested state contractions in concentrations which greatly reduce contraction strength at higher frequencies (Koch-Weser et al., 1964).

Koch-Weser and Blinks remained very cautious about proposing mechanisms which might account for the PIEA and NIEA. Not enough was known about the cellular processes involved in the heart contraction cycle, and for this reason no serious proposals could be made up to the middle of the 1960's. Important advances toward a better ionic and biochemical understanding of the heart were just beginning. None-the-less, biochemists were quick to see consequences of their work. In 1965 Robison et al. published biochemical work which implicated cyclc 3': 5'-adenosine monophosphate (cAMP) as the

"second messenger" mediating effects of epinephrine on the heart. These biochemists were struck by the effect of adrenergic stimulation on the PIEA as analyzed by Koch-Weser and Blinks; if epinephrine increases the PIEA per beat, and cAMP mediates the effects of epinephrine, it would be logical to conclude that the cAMP system must in some way be involved in the production of the PIEA. They suggested that the "enzyme or enzyme system" on which cAMP acts could be responsible for the production of the PIEA.

The basic machinery of the cAMP system is now widely thought to involve the following enzymes: Adenylate cyclase, the enzyme which produces cAMP from ATP; phosphodiesterases, which hydrolyze cAMP to the inactive AMP; protein kinases, which bind cAMP for activation and phosphorylate specific proteins with the gamma-phosphate of ATP; and finally, phosphoprotein phosphatases, which dephosphorylate the protein kinase substrates. The phosphorylation of specific protein kinase substrates is thought to be the final link between hormone receptor activation and effect (Walsh , 1978). According to this scheme of things the "enzyme or enzyme system" of Robison et al. would be cAMP-regulated protein kinase.

There presently exists substantial evidence that most or all of the effects of ß-adrenergic stimulation in heart are mediated by the cAMP system (Tsien, 1977). Substances which inhibit cAMP phosphodiesterase activity can mimic and enhance the effects of ß-adrenergic stimulation. cAMP derivatives which pass the cell membrane can mimic the effects of ß-adrenergic stimulation. Protein kinase activity in tissue samples increases under many conditions in parallel to effects of ß-stimulation. Finally, techniques have been developed in the last few years to measure phosphorylation of protein kinase substrates in intact tissues during hormone response. Methods for measuring phosphorylation of substrates in heart, thought to be involved in the positive inotropic effect of ß-adrenergic stimulation, are currently being tested (Walsh et al., 1979). At present there exists no viable alternative to account for how the ß-adrenergic receptor could be linked to a number of effects.

In spite of the body of evidence supporting the "cAMP-

second messenger hypothesis" and its quite wide acceptance, the suggestion of Robison et al., that the PIEA might have something to do with the cAMP system, has received practically no attention up to the present work. There are two main reasons for this neglect. First, new developments toward an understanding of the role of some ions in regulating contraction shifted interest strongly toward purely ionic mechanisms as explanations for the interval-strength phenomena. These considerations have largely eclipsed other possibilities from the discussion until quite recently. Second, the basic phenomenon of frequency inotropy (increase of contraction strength with increase of frequency) is proving to be a heterogenic group of effects in various heart muscle preparations. Blinks'and Koch-Weser's original proposal of one PIEA for all heart muscle types has simply not been borne out by experimental work in the last 10 years. Even in the same preparation more than one mechanism of frequency inotropy production may exist and be operative. This added complexity has made a differentiated discussion of the topic extremely difficult, sometimes impossible. The rest of this introduction is intended to elaborate on these two points.

It has been known since the beginnings of heart physiology that isolated heart muscle preparations depend on calcium in the bath solution for maintenance of contractility (Ringer et al., 1887). In the late 1940's and early 1950's a number of investigations implicated an important role for calcium ions in activation of muscle contraction (see Weber, 1976). By the middle of the 1960's it was clear beyond any reasonable doubt that muscle contractions are activated by a transient increase of the free calcium concentration in the cytosol. This realization posed many of the central problems of molecular cardiology right up to the present: From where and how is calcium released for activation of contraction? To where and how is calcium taken up for relaxation? How are these processes controlled in a muscle which behaves in an "all-or-none" fashion to efficiently regulate the heart beat under various demands and conditions? Finally, how can these processes be influenced by drugs? Any attempt to solve the puzzle of the interval-strength relationship has to deal with these questions. Since the heart is subject

to the influence of many drugs and hormones, this problem complex has often been taken up by pharmacologists. A dialectic is set up between what is known about processes controlling the heart contraction and what is known about the actions of a drug. As more is learned about the cellular function of the heart, more exact question can be posed about the action of a drug on the heart. When more is known about a drug action, this drug can be used as a tool to ask more precise questions about the cellular mechanisms of the interval-strength relationship.

The arguementation of Robison et al. for a cAMP-dependent mechanism of frequency inotropy production is an example of this approach. Another good example is the arguementation of Reiter and coworkers (Seibel, 1975; Ebner et al., 1977 and 1979) that an intracellular accumulation of sodium is the crutial process leading to frequency inotropy production in guinea pig papillary muscle. It is well-accepted that the intracellular sodium concentration is an important determinant of the degree of heart activation. Heart glycosides are generally thought to exert their positive inotropic action by inhibiting the sodium-potassium pump in the sarcolemma (muscle plasma membrane), resulting in an increased intracellular sodium concentration. In contrast to atrial muscle, the positive inotropic effect of heart glycosides in guinea pig papillary muscle is strongly frequency-dependent. Heart glycosides enhance frequency inotropy production, and thereby shift its accumulation to lower frequencies. Furthermore, the rate of accumulation and decay of frequency inotropy is slowed by heart glycosides. This behavior fits excellently with the idea that sodium accumulates in this preparation with increase of frequency, that this accumulation is coupled to a positive inotropic effect, and that the rate of decay of this effect is determined by the activity of the sodium-potassium pump.

Shelburne et al. (1967) have presented more direct evidence that in rabbit ventricle intracellular sodium may accumulate with increase of frequency. Potassium ion measurements would indicate that a sodium-potassium pump lag developes with increase of frequency. Sodium would accumulate intracellularly,

and a positive inotropic effect should develope via exchange of intracellular sodium for extracellular calcium. According to these authors sodium-calcium exchange could be beat-dependent and create a calcium influx per beat large enough to contribute directly to muscle activation.

Although evidence for a sodium-linked mechanism of frequency inotropy production in mammalian ventricle seems quite good, there is little support for such a mechanism in other preparations. Chapman and Niedergerke (1970) have argued against such a mechanism in frog heart on the basis of potassium loss and uptake kinetics with changes of frequency. As pointed out earlier, the positive inotropic effect of heart glycosides in mammalian atrium is independent of frequency, and the rate of decay of frequency inotropy is not slowed (Koch-Weser et al., 1964). Although evidence exists that intracellular sodium may accumulate in isolated atria during stimulation at extremely high frequencies (Glitsch, 1973), it seems unlikely that in this preparation a marked accumulation of intracellular sodium occurrs in the frequency range where frequency inotropy production is normally manifest. What mechanisms might regulate the intracellular sodium concentration in such a way that it does not accumulate to a large degree with increase of frequency?

Of the mechanisms proposed to date to account for frequency inotropy production, the hypothesis which has received the most attention revolves around considerations of how calcium is released for activation of contraction. It is well-known that calcium enters the cell with each beat via so-called "slow channels" in heart. The channels can carry both sodium and calcium (Vitek et al., 1971), and the plateau of the cardiac action potential is considered to be largely due to a calcium current through these channels (Reuter, 1967). Slow calcium channels are thought to exist in both an activatable and inactivatable state; stimulation of the cAMP system results in the recruitment of more activatable slow channels, possibly by a phosphorylation mechanism (Reuter, 1974; Reuter et al., 1976; Sperelakis et al., 1976). The main mechanism by which ß-adrenergic stimulation exerts its positive inotropic effect is probably a

cAMP-dependent increase of slow calcium inward current (Beresewicz et al., 1977). Estimates of the size of the slow calcium current fall short, however, of accounting for the amount of calcium needed for activation of contraction or the positive inotropic effect of ß-adrenergic stimulation (for example, Beresewicz et al., 1977), at least in mammalian ventricle preparations. For mammalian atrium no estimates of the size of the slow calcium inward current are available. Voltage clamp studies have proven to be especially problematic on atrial muscle, and only two such studies are known to me (Eick et al., 1976; Schulze, 1978).

It is also well-known that mammalian heart cells contain an organized tubular system, the sarcoplasmic reticulum (SR), capable of rapidly sequestering calcium via an ATP-driven calcium pump. It has been proposed and quite widely accepted that the SR releases calcium for activation of contraction in heart muscle (Fabiato et al., 1977) as well as skeletal muscle (Endo, 1977). From this perspective the role of slow inward current might not be to activate contraction, but to prime the intracellular calcium store. If the calcium entering the cell with each beat is taken up by the SR, together with the calcium just released for that beat, the possibility is given that the amount of calcium available for release will increase with increase of frequency. An intracellular accumulation of calcium would take place whenever the amount of calcium lost from the store from one beat to the next is less than the amount of calcium gained by the store via calcium entrence at the sarcolemma.

Before considering possible evidence for this hypothesis, in particular for atrial muscle, it seems important to point out that such a mechanism of frequency inotropy would not exclude the existence of other mechanisms. Frog heart contains very little SR, and Anderson et al. (1977) have presented evidence that activator calcium in frog enters the cell with each beat. Never-the-less, the frog heart shows frequency inotropy production to the same extent as other heart preparations. Chesnais et al. (1978) have presented strong direct evidence that the mechanism of frequency inotropy production in frog ventricle must result in an in-

ceased calcium influx per beat, and not an increased intracellular release (see Anderson et al., 1976 for a consideration of possible correlates between cardiac structure and interval-strength phenomena in various preparations). Niedergerke and Page (1977) have proposed that a beat-dependent phosphorylation of slow calcium channels may be an important mechanism of frequency inotropy production in frog atrium. We independently made a similar proposal for guinea pig atrium (Hilgemann et al., 1977). For frog atrium Noble et al. (1979) have reported an increased slow inward current with increase of frequency.

^{45}Ca flux measurements might be expected to give a good indication about the mechanism of frequency inotropy production. If guinea pig atrium would function like frog heart, a large calcium influx per beat would be expected--enough to activate contraction--, and the influx per beat would be expected to increase with increase of frequency. If an intracellular accumulation of calcium in an intracellular store were the main mechanism of frequency inotropy production, a small influx per beat would be expected, and the amount of exchangable calcium should increase with increase of frequency.

Unfortunately, calcium flux measurements on isolated heart preparations are not unproblematic. First, there is no way to identify the fluxes being examined with a certain structure or functional compartment. Second, the amount of exchangeable calcium in such a measurement is very large; related to wet weight it is usually more than 50% of the bath calcium concentration. Only a very small fraction of this calcium can be expected to be important for maintenance of contractility. Third, apparent calcium influx per beat as calculated from such studies may have little relation to the actual calcium influx per beat. If there is a rapid exchange into and out of the calcium pool involved in activation of contraction, the apparent calcium influx per beat may be a large underestimation of the real influx per beat.

As might be expected from these complications, ^{45}Ca flux measurements have been used to support both of the above

hypothetical mechanisms. For guinea pig atrium Winegrad and Shanes (1962) reported an increased influx per beat with increase of frequency. This work is subject to the criticism that the exchange pool being measured showed a very slow exchange rate and probably has no clear relation to contractility. Grossman and Furchgott (1964) considered exchange of the "contraction pool" to be very rapid and beat-dependent. Therefore, exchange measurements for various frequencies could only reflect changes of the influx per beat up to about 30 beats per minute. At higher frequencies pool saturation would be so fast that exchange values for various rates of contraction would be invalid. Accordingly, these authors considered their finding of an increased influx per beat up to 0.5 Hz to be real, and the apparent decrease of the influx per beat above 0.5 Hz to be due to limits of the method.

Other reports for atrium neither support or contradict the above findings. Little et al. (1969) reported no increase of influx per beat in guinea pig atrium between 1 and 2 Hz at 27°C. The main accumulation of frequency inotropy under these conditions takes place between 0.1 and 1.0 Hz, and above 1 Hz only transient increases of force are found (the authors' own curves and description). Haake et al. (1970) reported a decrease of the influx per beat with increase of frequency in guinea pig atrium. The calculations given are for values at close to saturation (30 min exchange), so that the decreased influx per beat with increase of frequency very probably reflects the problem of pool saturation pointed out by Grossman and Furchgott.

Of the studies known to me for atrial muscle, only Teiger and Farah (1967) reported a clear increase of total calcium with increase of frequency. This increase is probably not related to the mechanism of frequency inotropy production, since it could not be readily reversed by decreasing frequency. The effect more likely represents deleterious side-effects of the high frequencies employed in the study.

A plausible hypothesis can be validated for further consideration if it is shown that it accounts well for certain phenomena, which cannot be described by other available ideas. The phenemena of the interval-strength relationship are well-suited to this approach, and a number of simulated models of excitation-contraction coupling in heart have been developed (Manring, 1970 and Manring et al., 1971; Opitz, 1973; Wussling et al., 1973; Kaufmann et al., 1974). Each of these models assumes that the mechanism of frequency inotropy production is an intracellular accumulation of calcium. Most interval-strength phenomena are explained on the basis of intracellular calcium movements. Only one example is known to me, where the question was asked, if, and not how a certain effect could be explained by calcium movements or accumulation in an intracellular store. After attempting to mathematically model certain of the rapid effects of interval change in rabbit ventricle, Johnson et al. (1971) commented that these effects could not be explained by "changes in the amount of calcium sequestered in some compartment or site from which it is released on excitation--either calcium has nothing to do with the changes in contractility between beats or what changes between beats is, for example, the permeability or binding constant of some membrane through which calcium flows or from which it is released when the cell becomes electrically activated."

Of the simulated models developed to date, Manring (1970, 1971) dealt in most detail with atrial muscle. He developed a two-compartment model for calcium movements which produces some of the atrial interval-strength phenomena of guinea pig atrium. Step changes of frequency, for example, result in "biphasic" changes of contraction strength roughly similar to real muscle. The model does not predict the fall of steady state contraction force with increase of frequency from the rested state. The steady state frequency-force relationships produced bear little resemblance to the real relationship, even in the Bowditich part of the relationship. Pharmacological interventions were not simulated. The claim is made to incorporate the calcium flux measurements of Winegrad and

Shanes (1962) and Grossman and Furchgott (1964) into the model. None-the-less, the findings of these authors of an increased calcium influx per beat with increase of frequency has no counterpart in the simulated model. Other authors dealt only in-passing with the atrial interval-strength relationship.

The most elegant and detailed simulated model developed to date is an analogue computer model for papillary muscle of kitten (Kaufmann et al., 1974). This model produces both an action potential and contraction analogue. The rapid effects of interval change seen in this preparation under the conditions studied are accurately reproduced, and the effects of calcium and several negative inotropic agents were simulated. It is convincingly demonstrated that if the assumptions going into the model are correct, then a number of interval-strength phenomena will be explained by calcium movements and accumulation in the electromechanical coupling system. The slower effects of interval change found in ventricular preparations are not simulated.

Certainly, many aspects of the simulated models developed to date will prove to be correct. But it seems fair to conclude that none of these models can deal with the atrial interval-strength relationship. The atrial "PIEA" behaves very differently than frequency inotropy as it occurrs in the model of Kaufmann et al. PIEA decay is very slow in comparison, its rate of decay does not depend on the rate of stimulation, and the Bowditch part of the atrial frequency-force relationship is much steeper than curves given for kitten papillary muscle. Whether these differences are only quantitative in nature, or also qualitative, is an open question. No model has been shown to accurately predict the frequency-force relationship of atrium with Kruta and Bowditich parts.

One of the major assumptions made in the above simulations is still open to debate, the assumption that calcium is released from the sarcoplasmic reticulum to the cytosol for activation of contraction in heart. Another solution to the problem of contraction activation in hearthas been proposed, which does not need to assume an intracellular calcium release. Langer et al. (1976) have proposed that

an "essentially electroneutral" sodium-calcium exchange mechanism could rapidly move calcium into the cell at depolarization. Such an exchange, together with the slow calcium inward current, could supply enough calcium for activation of the contractile proteins without an additional release from intracellular stores. It seems prudent to remain open about the immediate source of activator calcium. Experimental difficulties in demonstrating an intracellular release of calcium for contraction activation could reside in the inherent methodological problems of such a proof. As well, it remains a possibility that the mechanism for which a proof is needed does not exist. Assumptions other than those used for simulations to date, or certain modifications of those assumptions, might describe the interval-strength relationship of ventricle, as well as atrium, equally-well or better.

Thus, at the onset of this dissertation it seems fair and important to consider the interval-strength relationship of guinea pig atrium as a book with blank pages. We know that the essential mechanisms behind the relationship must result in changes of the amount of calcium activating contraction with each beat. There is indirect evidence against the idea that an intracellular accumulation of sodium could be the main mechanism of frequency inotropy production in atrium. There is circumstantial evidence that an intracellular accumulation of calcium could be involved in frequency inotropy production. There is a circumstantial reason to think that the cAMP system could be involved in the mechanism of frequency inotropy production in atrium. We know that intracellular structures in heart are capable of rapidly sequestering calcium, but we cannot be certain that calcium is directly released from these structures for activation. We know that a cAMP-sensitive slow inward current exists in heart, but we know little about its size in atrium. There is reason to think that the mechanism of frequency inotropy production in atrium leads to an increased calcium influx per beat. No hypothesis proposed to date can clearly account for the reduction of contractility in the low frequency range in atrium. We have a lot more building blocks and pharmacological tools than just a few years ago. It is

hoped that these means will be enough to come closer to an understanding of the atrial interval-strength relationship. That is the goal of this dissertation.

2. Methods.

2.1. Muscle preparation, muscle chambers, muscle stimulation and contraction registration§.

Young male guinea pigs (160 to 240 g) were used for all experiments presented. Larger animals were not taken to limit the thickness of muscle preparations (0.4 to 0.7 mm for both atrial walls taken together). Animals were killed by a shot to the base of the skull with a spring-loaded gun, which upon triggering ejects a small metal rod of 6 mm diameter with a sharp ring edge. Hearts were rapidly removed and within 20 s after kill were placed in a small dish containing modified Krebs-Henseleit solution (32°C, bubbled with 95% O_2/5% CO_2; see 2.2 for exact composition) with 3×10^{-4} mol/L adenosine. This concentration adenosine reduces the spontaneous rate and contraction force of guinea pig atria within seconds to very low levels. This intervention insures that the atria are not metabolically stressed by the high spontaneous frequency. I prefer this pharmacological intervention to dissection in cold solution, since "cold" is not without long-term effects on atrial preparations (unpublished observations).

The left atrium is cut away from the heart and removed to a special dissection chamber (no adenosine), which is designed to recirculate oxygenated, temperated bath solution rapidly through the dissection area. The tissue immediately surrounding the atrial-ventricular valve is carefully cut away to insure bath solution exchange to the atrial lumen during experiments. The atrium is fixated at this end in a small wire clamp with wire connection for force transfer to the transducer system. The other end of the preparation is fixated by a wire mechanism in a plexiglass muscle holder, and the holder with muscle preparation is fitted into a special muscle chamber system for functional experiments. The muscle preparations weigh from 15 to 30 mg and are thin enough to be translucent through both atrial walls.

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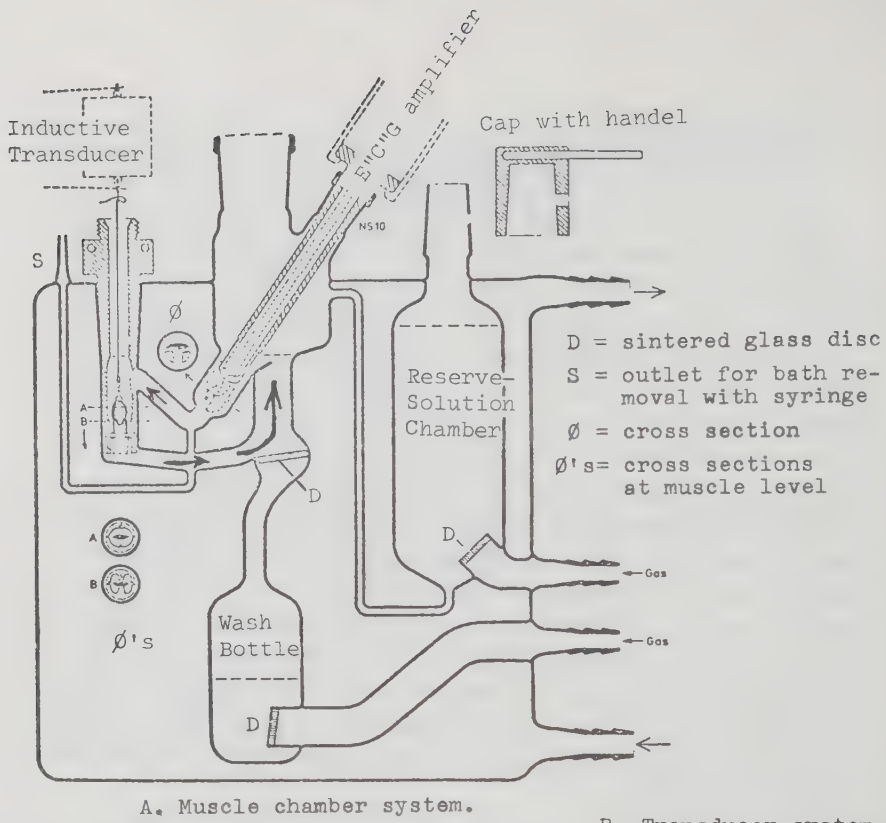
§ The muscle chamber systems were designed by Mensing and built by Glasinstumentenmachermeister Jung in Marburg. The muscle holder was designed by Mensing and Stieler and built by Stieler in this institute. The transducer system was designed by Mensing and built by Stieler in this institute. See Mensing (1977) for a detailed discussion of the bath method for investigations on isolated heart preparations.



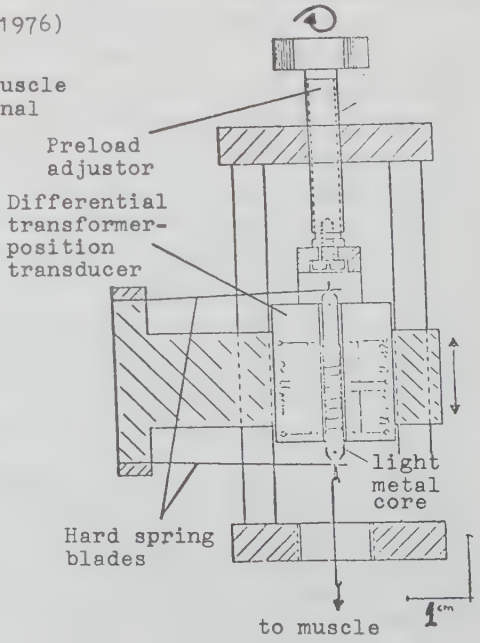
A. Muscle chamber system.

B. Transducer system.

Figure 2.1 (from Mensing, 1976)

to A:
Schematic diagram of the muscle chamber system for functional experiments.
Reserve bath solution can be quickly forced into the muscle chamber by gas pressure when the cap with handel is closed.

to B:
Schematic diagram of the transducer system.
Fine preload adjustment is possible via the fine winding of the adjustor shaft.



The muscle chamber system used in functional experiments is diagrammed schematically in figure 2.1. The system is made entirely of glass, so that it can be cleaned in acid and sterilized in an oven. Gas is first bubbled through a washing bottle for temperation and saturation with water. It then bubbles through the actual muscle chamber, and the rising of bubbles drives the bath solution through the chamber and directly past the muscle. This rapid recirculation of of bath solution prevents formation of an unstirred solution layer at the muscle surface. Spacial removal of the bubbling from the muscle itself insures that bubbles will not disturb force measurement. The chamber needs just 10 ml solution for the recirculation system to operate with the muscle holder fitted into the chamber. A newer version of the chamber system functions with only 3 ml, a great advantage if experiments are made with expensive substances. Bath solution can be quickly removed from the muscle chamber with a syringe. Fresh, oxygenated bath solution can be forced out of the reserve chamber within the same water jacket and into the muscle chamber. In this way the bath solution in the muscle chamber can be comfortably exchanged within 6 to 12 s. The ECG-amplifier with plexiglass holder, shown in the figure, is for registration of the spontaneous frequency of the right atrium. This was not used in these experiments.

The plexiglass muscle holder used for these experiments is designed in two halves. The muscle is fixated in one half by a wire mechanism which presses the lower edge of the atrium against a small platinum electrode, poled as cathode for muscle stimulation. The electrical connection to the top of the holder is isolated from the bath solution. The second half of the holder contains a larger electrode which is not brought in contact with the muscle and is poled as anode. The electrode design allows use of the smallest possible electrical impulses for muscle stimulation. The stimulation current is always set at just above contraction threshhold at 1 Hz. With increase of frequency the stimulation threshhold shifts slightly upward. Stimulation current needed at 0.1 ms amounts to 0.3 to 1.0 mA; apparent muscle resistance is about 2 kOhm. For "slow response" experiments (after inactivation of fast sodium channels) a different muscle holder is used which contains large electrodes

situated on each side of the muscle for field stimulation.

Endogenous catecholamine release can be a problem working with atrium. It is important to keep in mind that the threshhold for catecholamine release by electrical stimulation is not far from the threshhold for contraction. I find a detectable positive inotropic effect by doubling either the impulse time or current. However, concentrations of propranolol which block this effect have little or no negative inotropic action and do not change any of the interval-strength phenomena described in this dissertation (see Koch-Weser, 1965, for a more comprehensive consideration of this problem).

In our experimental set-up the transducer system is mounted directly on top of the muscle holder. A differential transformer-position transducer (TRANS-TEK, type 240000) is employed with a light moveable core between two hard spring blades. Resonant frequency with the muscle connected is about 80 Hz. Compliance is 0.1 mm/10 mN. Contraction can be considered isometric; the largest contractions recorded result in about a 4% shortening of the muscle.

The contraction signal is processed electronically (Siess et al., 1970) for continuous registration of contraction parameters on a multipen recorder. The following contraction parameters can be routinely recorded; maximal velocity of contraction or relaxation, time to peak or time to relaxation (with a variable threshhold), and peak contraction force. In addition the contraction signal is followed visually on an oscilloscope. A typical contraction and its first derivative ($32^{o}C$, 1.0 Hz) is shown in figure 2.2. In atrium, the time of relaxation is nearly always somewhat longer than the time to peak. Most inotropic interventions result in quite symetrical changes of the contraction curve.

A preload of 7.2 to 7.5 mN was used in all experiments presented. This preload represents points close to, but never more than, the maximum of the length-force relationship for the preparations.

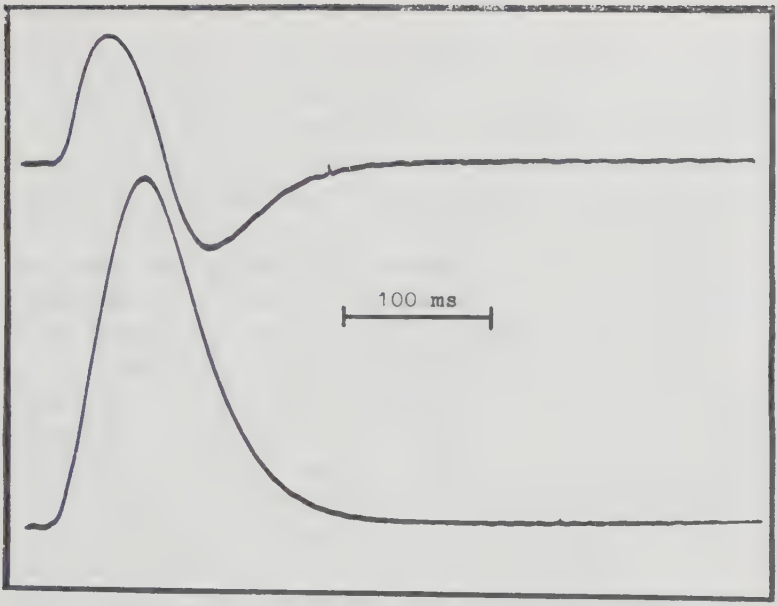


Figure 2.2. A representative contraction curve and its first derivative (32°C, 1.0 Hz, 2.5 mM calcium). The derivative curve runs a few milliseconds to the right of the contraction curve.

Although changes of time to peak and time of relaxation with inotropic interventions are much less marked in atrium than in ventricle, the following changes can be reported with reference to inotropic effects studied in this dissertation:

1) ß-adrenergic stimulation can shorten the entire contraction by 15 to 30%, with a larger effect on time of relaxation than time to peak. This change is not reversed by purinergic stimulation, reduction of the bath calcium or by nifedipine.

2) Temperature reduction markedly lengthens the contraction time from around 110 ms at 37°C to 230 ms at 27°C (2% force development to 98% relaxation).

3) For the quickest, one-beat effects of interval change the maximal velocity of relaxation does not increase (or decrease) parallel to the maximal force development; for example, in a step reduction of frequency the maximal tension increases in the firstbeat, but the relaxation velocity increases very little or even remains unchanged.

4) After ryanodine treatment the rested state contraction shows a slowed onset of force development.

2.2 Bath solution and drugs.

A modified Krebs-Henseleit solution was used as bath solution for all experiments presented: 120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 or 1.2 mM CaCl as given in figures, and 15 mM glucose. Equilibriated with 95% O_2/5% CO_2 this solution gives pH 7.4 at 30 to 35°C.

To be able to assess accurately negative and positive inotropic effects, it is important to keep force development in the intermediate range of contractility. At 32°C and in the frequency range chosen to avoid metabolic fatigue (less than 1.5 Hz), this preparation developes too little contractile strength with 1.2 mM calcium to assess negative inotropic effects. Therefore, 2.5 mM calcium is used at 32°C. This represents about twice the "physiological"value (Blaustein, 1974).

Drugs are added to the muscle bath from stock solutions with microsyringes. The following substances were used:

Adenosine; Merck

Adenosine- 5'- triphosphoric acid disodium salt (ATP); Merck

8-Benzylthio-adenosine 3': 5'-cyclic monophosphate; Gift of Dr. H.J. Wachtel (Berlin)

Carbachol (Doryl^R); Merck

2,4-Dinitrophenol; Leopold Cassela and Co.

3-Isobutyl-1-methyl-xanthine (IBMX); SIGMA

Dipyridamole (Persantin^R); Thomae

Nifedipine; Gift of the company Bayer.

Ryanodine; A generous donation of Dr. Price, Merck Sharp & Dohme.

Tetrodotoxin (TTX); Sankyo

g-Strophanthin (oubain); Merck

Veratridine; A kind gift of Dr P. Honerjäger (Münich)

2.3 Curve-fitting and simulation.

The versatile curve-fitting and -plotting program used in this dissertation was written by R. Englert in this institute. All curve-fitting was done by the least-squares method. Both curve-fitting and curve simulations were done on a Hewlett-Packard desk computer (9820A) with plotter and expanded storage.

3. Automated frequency-force relationships.

Frequency-force relationships presented in this paper were made with a method developed by Mensing, Englert and Hilgemann in 1975-76 in this institute. This method utilizes a special frequency generator to change continuously the frequency with which an atrial preparation is driven from a chosen lower limit, usually 0.005 Hz, to an upper limit, usually 1.5 Hz, according to an approximated exponential function ($f = f_o e^{\pm kt}$). When the upper limit is reached, frequency change is reversed toward the lower limit with the same function (see figure 3.1). On an X-Y recorder the frequency being generated (X-axis) is recorded directly against the electronically processed signal for maximal force development (Y-axis). The rate of frequency change is set slow enough ($k = 0.004\ s^{-1}$) so that the inotropic effects of frequency change reach nearly steady state at all frequencies. The curve given in figure 3.2 is typical for the relationship at 2.5 mM calcium and 32°C.

Two factors were of primary importance in choosing an exponential function for frequency change: 1) Both the Kruta and the Bowditch parts of the frequency-force relationship should be well characterized. 2) Frequency-force relationships should be established as quickly as possible with still maintaining nearly steady state conditions.

The accumulation of NIEA in the Kruta part of the relationship is beat-dependent. For this effect to reach a certain fraction of its steady-state level with frequency change, a certain number of beats must be made, regardless of frequency. The NIEA decays between beats with a certain time constant, but it always needs 3 to 6 beats to accumulate. Therefore, the same number of beats should be made for equal changes of frequency to allow this effect to reach nearly steady state with continual frequency change. This relationship is given by the exponential frequency change (see figure 3.3 for an exact explanation).

As demonstrated by Blinks and Koch-Weser, the PIEA behaves on the whole like a simple, linear process. That is, a small amount of this effect is produced with each beat, and it decays exponentially between beats. A <u>linear</u> change of frequency over time might therefore be advantagous for the PIEA, since equal time units would be given for accumulation

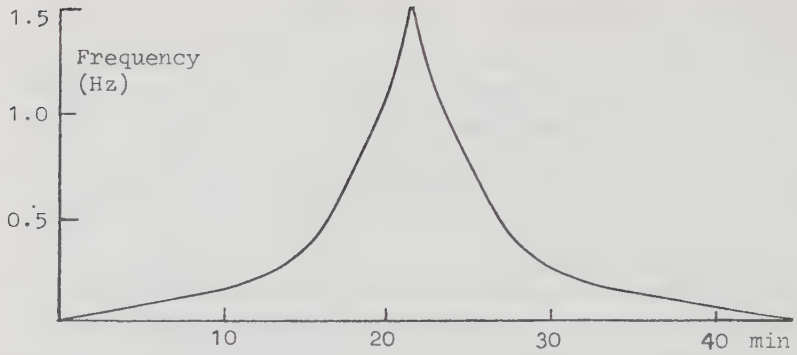


Figure 3.1. The frequency change produced by the frequency generator over time with the usual rate setting (t_{50} = 3 min).

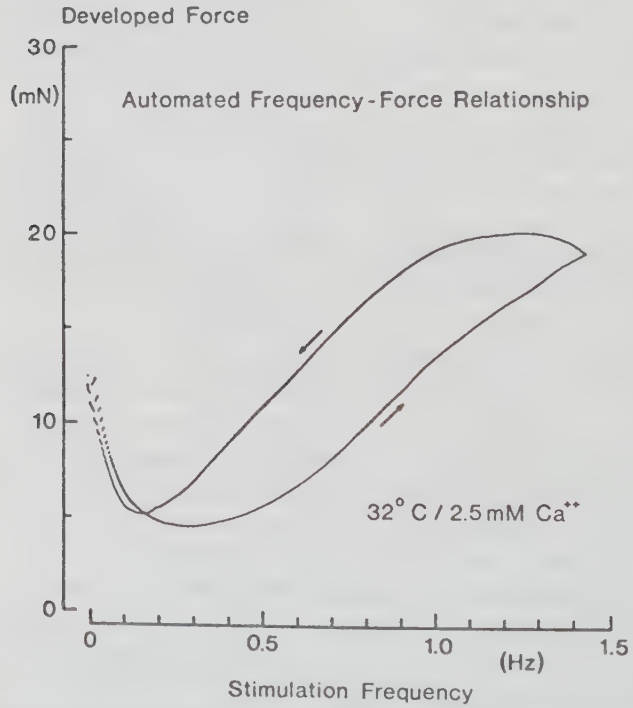


Figure 3.2. A representative frequency-force relationship as it is depicted by the automated method. 32° C; 2.5 mM calcium. Arrows indicate the direction of automatic frequency change. Zig-zags in the lowest frequency range correspond to an upward drift of the peak force signal between beats. See text for further explanations.

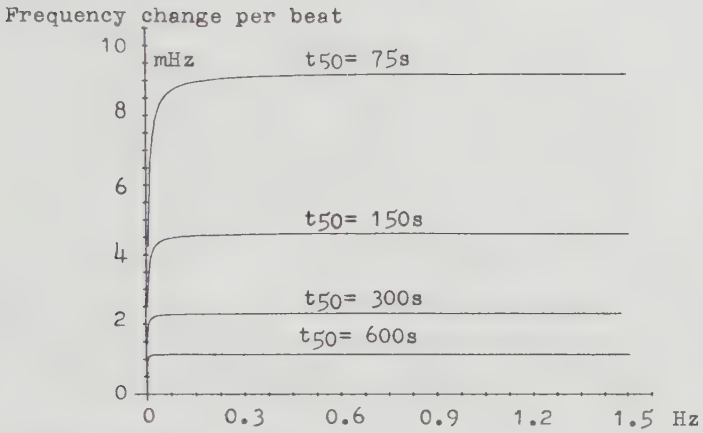


Figure 3.3. The figure demonstrates the frequency change per beat made for exponential frequency change at various rates. Above a quite low frequency range the frequency is increased by the same amount from one beat to the next. The change of frequency per beat (Hz = s^{-1}) turns out to be equal to the rate constant in the equation for frequency change:

$$f = f_o \cdot e^{\pm k \cdot t}$$

f= frequency
k= $\ln 2 / t_{50}$ for frequency change.

Now let f_o be the frequency of any beat and f be the frequency of the subsequent beat. An impulse will be given off by the frequency generator when the interval between beats, t, is equal to 1/f. For this situation:

$$f = f_o \cdot e^{\pm k/f} .$$

The above curves were obtained by solving this equation by a Newton procedure on a beat-to-beat basis.

For the frequency range where frequency change from one beat to the next is nearly constant, the above equation can be simplified to:

$$f - f_o = k \quad \text{(valid for the frequency range where, } e^{k/f} < 0.1\text{)}.$$

For simulation of various effects, this simpification is accepted, and the frequency is changed by equal amounts from one"beat"to the next for the entire frequency range.

of equal amounts of PIEA. Figure 3.4 contrasts in simulated curves how a simple, linearly accumulating effect would be represented with a linear and with an exponential change of frequency. For the linear frequency change the rising and falling curve parts lie parallel to each other and to the real steady-state line. For exponential frequency change the rising and falling parts of the curve form an angle above and below the steady state line. An advantage of the exponential frequency change is that the loop formed is closed at the bottom. A disadvantage is that the slope of the rising part of the loop is not identical to the slope of the steady state line.

One practical advantage of changing the frequency exponentially is that preparations need to beat at frequencies above 1.0 Hz for only short time periods. In this way the effects of metabolic fatigue can be, at least for the most part, avoided. This is an important consideration, since we are interested in the real mechanisms responsible for frequency inotropy production. My experience is that preparations begin to show characteristic, progressive negative inotropic effects even at 1 Hz. Particularly at temperatures above 32°C, even a short-term exposure to stimulation above 1.5 Hz can result in a negative inotropic effect from which a preparation recovers only very slowly,if at all, in the low frequency range. Thus, the exponential frequency change allows a good look at the mechanism of frequency inotropy over a larger range than otherwise would be possible on an isolated, non-perfused preparation. If a frequency of 1.5 Hz is not exceeded, consecutive frequency-force relationships are quite stable; in the course of 5 h (32°C) contraction force under control conditions deteriorates by about 50%. The maximal contraction force attained by a preparation with various stimulants or combinations of stimulants (ß-adrenergic stimulation, high bath calcium, and heart glycosides) shows little or no change even after 24 h.

Two clear advantages of the automated method are a facilitation and standardization of the quite tedious task of manually changing the driving frequency and plotting the results by hand. Without this aid it would not have

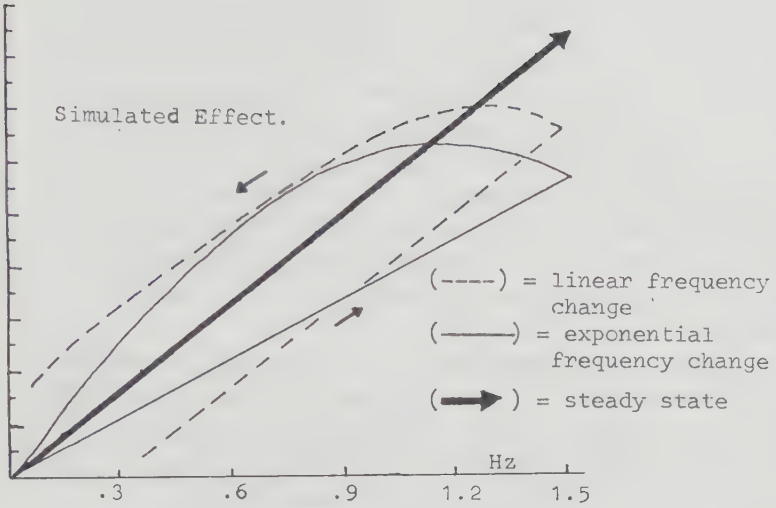


Figure 3.4. The figure contrasts how a linear effect would be depicted with an exponential and with a linear frequency change. With each "beat" a certain amount of an hypothetical effect is produced; between beats the effect decays exponentially.
The width of loops is determined by the relation of the rate of frequency change to the rate of decay of the effect. Increasing the speed of frequency change increases the width of the loop, but the loop would still rise and fall around the same steady state line. This effect results in a change of loop form identical to the difference between loops 3 and 2 in figure 3.5.
For the curve with exponential frequency change in figure 3.4, the rate of decay of the simulated effect was set twice as fast as the rate of frequency change. This gives a point of reference for estimation of decay rates from real muscles.
In experiments a compromise must be made between the width of loops acceptable for estimation of steady state and the time needed to make the loops. If loops are made too slowly, the contraction force at one frequency may no longer correspond to the preparation's contractile condition at other frequencies; preparations are not infinitely stable. Furthermore, to study pharmacological interventions several consecutive curves must be similar under control conditions.

been possible to examine the frequency-force relationship under such a wide variety of conditions in the last 4 years. Also, the size of frequency-force loops formed can be used to give a rough estimate of the rate constant of the PIEA. Figure 3.5 contrasts the effect of a hypothetical positive inotropic effect due to an increase of the PIEA per beat with one due to a slowing of the PIEA decay. On the basis of the steady state frequency-force relationships these two effects would appear identical. With the automated method the different mechanisms of action would be obvious.

Note here, as in figure 3.4, that the steady state for a hypothetical PIEA is a straight line. This only results when the relationship is plotted in a linear frequency scale; and this is an important advantage of using the linear frequency scale. Many other authors plot the contraction force against a logarithmic frequency scale and some against a logarithmic interval scale. These two methods have the advantage of stretching out the lower part of the frequency-force relationship, and in atrium this gives a better look at the Kruta part of the relationship. However, the linear frequency scale allows an assessment of how well the production of frequency inotropy follows the behavior expected for a simple, linearly accumulating effect. In frequency-force loops the rising curve for increase of frequency is ideally a straight line in the Bowditch part.

Finally, it seems important to counter an arguement which has been leveled against this method a number of times: The method never allows the muscle preparation to come to a final steady state, and therefore the method only gives a rough approximation of the real frequency-force relationship. Just as well, the arguement can be brought against the manual method that it is subjective. The steady state value accepted by the experimenter may be just as much a measure of his patience as it is of the steady state of the muscle. Furthermore, it is possible to judge the stability of a muscle preparation via the relationship between the rising and falling parts of the

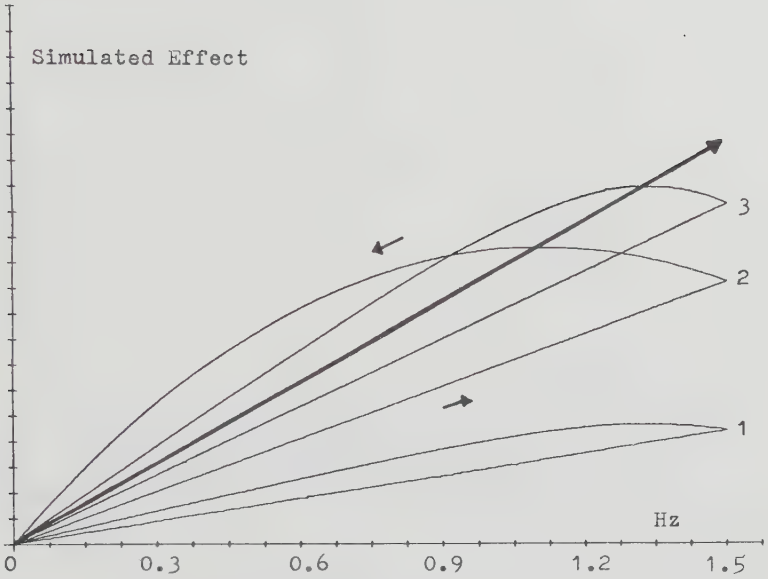


Figure 3.5. The figure contrasts how an increased PIEA/beat and a decreased rate of PIEA decay would hypothetically be depicted by the automated frequency-force method. From curve 1 to curve 2 the decay rate of the hypothetical PIEA was decreased by a factor 3. From curve 1 to curve 3 the hypothetical PIEA per beat was increased by a factor 3. For the curves 2 and 3 steady state contraction force would be identical (heavy line). The automated method would however reveal a clear difference between the two positive inotropic types.
Although these curves were made by calculating from one beat to the next (discrete simulation), identical curves are given by the following equations:

the steady state line is; $\frac{E_b \cdot f}{K_E}$

the lower loop part is; $\frac{E_b \cdot f}{K_E + K_f}$

the returning loop part is;

$$\frac{E_b \cdot f}{K_E - K_f} - \left(\frac{E_b \cdot f}{K_E - K_f} - \frac{E_b \cdot f}{K_E + K_f} \right) \cdot (f / f_{max})^{K_E / K_f}$$

E_b = effect per beat
K_E = rate constant of the effect
f = frequency
K_f = rate constant of frequency change
f_{max} = 1.5 Hz

The equations were derived by Laplace transformations and checked by comparison to simulated curves.

frequency-force loop. It is a seldom study in which it was said that the "steady state" values measured were re-checked a second time. Above frequencies around 1 Hz there is no "steady state," at least not in guinea pig atrium. Contraction force may come to a steady value over time, but this value reflects the added effect of metabolic fatigue--if only of the inner cells of the muscle preparation.

In one other respect, the automated method is also more exact than the manual method. By continually changing the frequency, all frequency ranges are examined, and nothing is left out. For example, with heart glycosides, high bath calcium or high bath temperature (or combinations of these interventions), force often rises with increase of frequency from the rested state, and then at higher frequencies the normal Kruta part of the relationship follows, followed by the Bowdich part (figure 8.10 in chapter 8). There exists under these conditions a small, but real maximum of the frequency-force relationship in a low frequency range. Although the conditions under which this maximum appears have been studied before, the maximum in the Kruta part of the relationship was not described. If the frequency is changed manually, such an effect can be easily passed over. Should there be a question that this maximum might be an artifact produced by the automated method, this possibility was clearly eliminated by examining the relationship by the manual method.

Figure 3.6 shows the function produced by the frequency generator, fitted to an exponential, and plotted semi-logarithmically. As is apparent, the frequency generator is not perfect. The greatest deviation of the approximated function from the ideal function occurs in the frequency range below 0.1 Hz. Between 0.005 and 0.05 Hz the frequency is changed much too quickly. This is apparent in frequency-force loops, in that the frequency steps between beats at the lowest frequencies are much too big. Although the frequency generator as-it-is was adequate for most purposes, the deviations apparent in figure 3.6 from the ideal exponential function make it difficult to exactly evaluate simulated relationships presented in chapter 8.

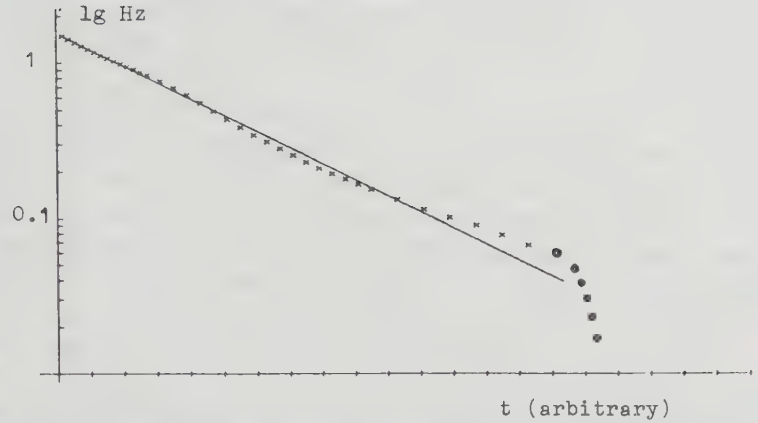


Figure 3.6. The figure shows deviations of the function produced by the frequency generator (x's & o's) from an ideal exponential (line). Points were fitted to an exponential and plotted semi-logarithmically. o's were not used for fit.

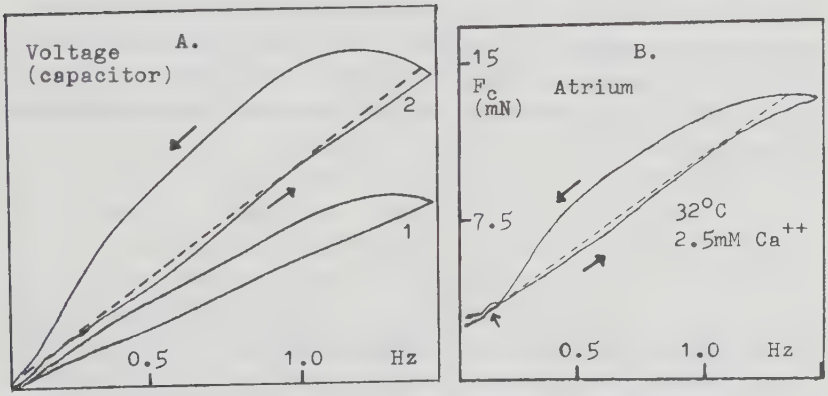


Figure 3.7. Curves in 3.7.A were made by charging a capacitor with the constant charge impulses from the frequency generator. From curve 1 to curve 2 a larger resistor was added in parallel to the capacitor. The curve in B is a frequency-force relationship from an atrium lacking rested state contractions (untypical). The same deviations from ideal curve form are apparent in A and B. An additional small deviation of the atrium, not due to the frequency generator, is marked by an arrow. The dotted lines are intended to accentuate the waves in the rising curve parts.

It is also difficult to evaluate finer aspects of the frequency-force loops, themselves, when there are necessarily deviations from ideal behavior in the curves. Unfortunately, it was not possible to better the method over the last four years.

In figure 3.7 the impulses produced by the frequency generator were used to charge a capacitor (the curves were made by Dr. Mensing). Note the deviations of the curves for the capacitor from the ideal curve form in figure 3.5. The rising part of the loop, which should be a straight line, shows a number of waves. Now compare the capacitor curves (figure 3.7.A) to the frequency-force loop from a real atrium in 3.7.B. Exactly the same deviations from ideal behavior are apparent. The atrium in 3.7.B is only a special case in that it showed no Kruta part. (I have had about 4 or 5 such cases in over 500 preparations studied.) Normal atria also show these deviations, but they are most obvious in such an atrium with only the Bowditch curve part. This example demonstrates how close indeed the behavior of frequency inotropy production can come to an ideal, linearly-accumulating process (= capacitor-resistor combination). Although the frequency generator is somewhat less ideal, **none of the effects on the frequency-force relationship presented in the next chapters can be explained by the deviations pointed out here.**

4. Abolition of the normal frequency-force relationship over a large frequency range by stimulation of the cAMP system and reduction of slow inward current.

4.1. Comparison of four negative inotropic actions.

The negative inotropic effects of four substances on the normal atrial frequency-force relationship ($32^{o}C$, 2.5 mM Ca^{++}) are presented in figure 4.1. Three of the substances have very similar effects under these conditions. Nifedipine, adenosine and carbachol all depress contraction force at high frequencies much greater than the rested state contractions. In terms of the Blinks and Koch-Weser analysis such an action must primarily reflect a reduction of PIEA accumulation. Since frequency-force loops do not become greatly narrower with the negative inotropic agents, their actions must be more a reduction of the PIEA produced per beat than an acceleration of the PIEA decay. The action of the naturally-occurring insecticide, ryanodine (Crosby, 1971), is different. As shown by Hajdu (1969) the atrial rested state contractions are quite completely depressed by ryanodine in concentrations which have only small or intermediate effects at higher frequencies. In contrast to the other substances, ryanodine has little or no effect on the slope of the rising part of the relationship.

Since the effects of carbachol, nifedipine and adenosine are so similar, an obvious question is raised: What might be the common denominator of their actions? The likely answer is that all three substances reduce the slow calcium inward current in atrium, although by partially different mechanisms. Carbachol (= cholinergic stimulation) and adenosine (= purinergic stimulation) both markedly shorten the action potential of guinea pig atrium (Johnson, 1956), and thereby would reduce the time for slow inward current to flow. For carbachol it is known that the action potential shortening is independent of contraction frequency (Ravens and Ziegler, 1979). For carbachol it is also known that the number of calcium channels available for opening can be reduced to some extent (Eick et al., 1976). Whether or not adenosine as well reduces the number of available slow channels is unknown. Belardinelli et al. (1979) have studied effects of adenosine on slow action potentials

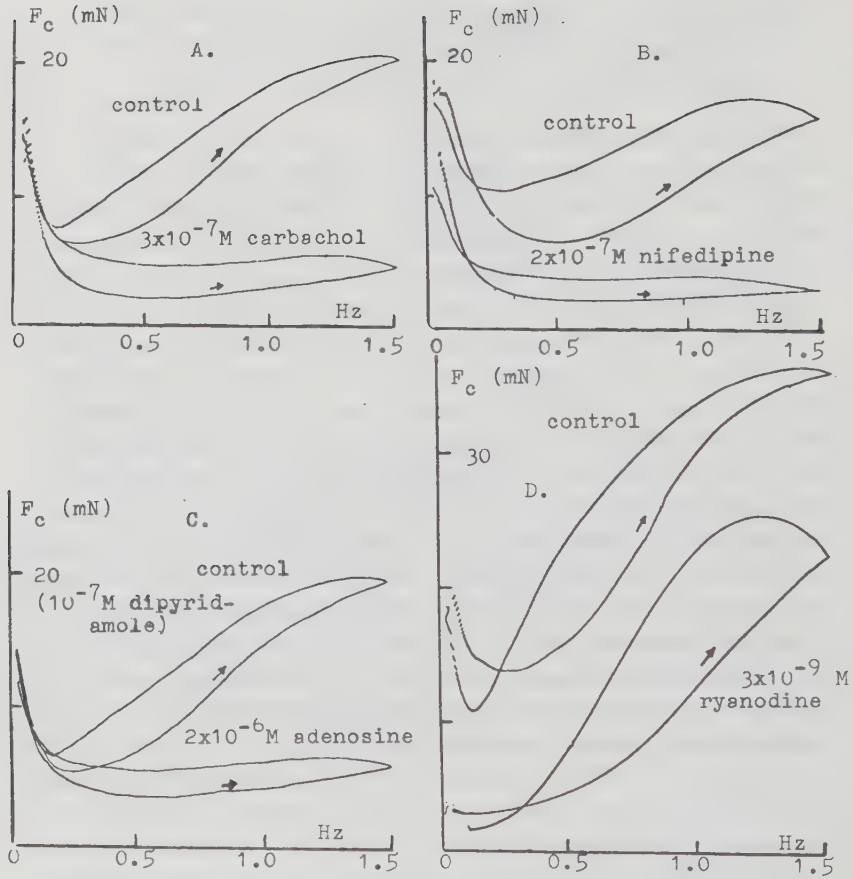


Figure 4.1. The figure contrasts representative negative inotropic effects of four substances on the frequency-force relationship. 32°C ; 2.5 mM Ca^{++}. Carbachol (A), nifedipine (B) and adenosine (C) all have quite similar effects; rested state contractions are little reduced by concentrations which have marked negative inotropic actions at higher frequencies. The effect of ryanodine is markedly different; it is the only substance known to greatly depress the atrial rested state contractions in concentrations which have only small or intermediate effects at high frequencies (D). The effect of $2x10^{-6}$M adenosine in C corresponds to the effect of about 10-4M adenosine without dipyridamole.

of rat strium and interpreted findings in this sense. Without voltage clamp studies, however, this question cannot be answered. Adenosine quite certainly increases potassium permeability (as does carbachol); and this effect with the accompanying action potential shortening would be enough to account for the negative inotropic effect (see Hartzell for a detailed study of adenine compounds and acetlycholine on slow inhibitory potentials in frog sinus venosus).

Nifedipine reduces the number of available slow channels and this action is independent of frequency, at least in ventricle (Ehara and Kaufmann, 1977). In contrast to other "calcium antagonists", the action of nifedipine on contractility as well reflects a "use-independence" (Rodenkirchen et al., 1980).

The next logical question is how a reduction of slow inward current might reduce the production or accumulation of frequency inotropy in atrium. A quite straight-forward answer might be given in the context of the hypothesis that the major mechanism of frequency inotropy is a simple accumulation of calcium in an intracellular store. A reduction of slow inward current would simply reduce the amount of calcium available for accumulation per beat. This would be just the opposite effect of ß-adrenergic stimulation, which increases slow inward current and therefore would increase the amount of calcium available for accumulation per beat.

Thus, this hypothesis predicts a functional antagonism between ß-adrenergic stimulation and the agents which reduce slow inward current. If ß-adrenergic stimulation is used to antagonize the effects of adenosine, nifedipine or carbachol, slow inward current should be increased again, and the frequency-force relationship should return to basically its normal form. The rest of this chapter documents the striking contradiction to such a prediction, which results by studying these interactions.§

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§ The interactions of adenosine with ß-adrenergic stimulation were published in a short paper (Hilgemann et al., 1977)

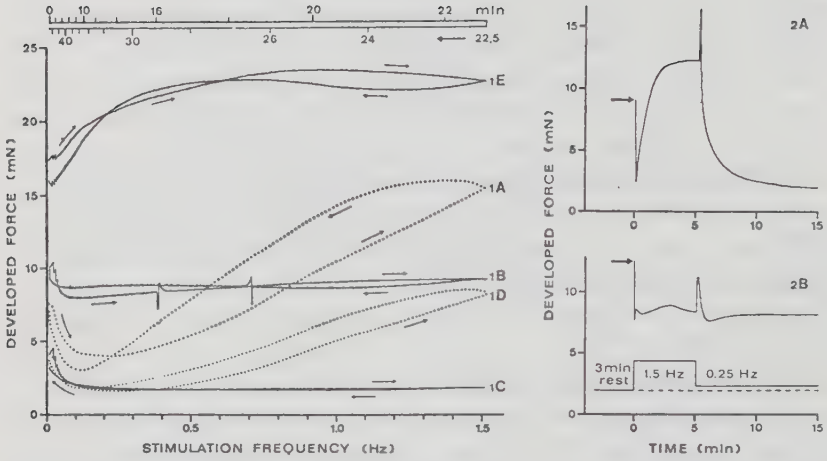

Figure 4.2. All curves from a single atrium. Curves 1B, 1C and 1.E are original registrations, 1A and 1D were retraced for clarity. 1A Initial control. 1B With 10^{-5}M orciprenaline antagonized by 3 x 10^{-4}M adenosine; two extrasystolic beats. 1C With 10^{-5}M orciprenaline plus 10^{-3}M adenosine. 1D Control curve after thourough drug wash-out. 1E With 10^{-5}M orciprenaline alone. In the right panel of the figure, curves 2A and 2B give force responses to step frequency change for the conditions of 1A and 1B, respectively. 32°C ; 2.5 mM Ca^{++}.

4.2. Interactions of adenosine and ß-adrenergic stimulation by orciprenaline.

The basic interactions of adenosine and orciprenaline[§] on the frequency-force relationship are presented in figure 4.2, characteristic for over 20 similar experiments. 1A is the initial control frequency-force loop. In 1B the nearly maximal ß-adrenergic stimulation by 10^{-5}M orciprenaline was antagonized by the negative inotropic effect of 3 x 10^{-4}M adenosine. With these opposed interventions contraction force is nearly independent of contraction frequency above 0.1 Hz. This frequency-force loop (1B) cuts through the control loop at an intermediate level, and more adenosine (10^{-3}M) displaces the curve downward in a quite parallel

[§] Orciprenaline is preferred to other ß-stimulants, since it is stable in solution.

manner (1C). After wash-out of drugs the second control curve (1D) shows about 50% of the initial force values (1A). This difference represents the normal effect of aging preparations at 32°C for about 5 h with continual electrical stimulation (Note: At lower temperatures preparations show markedly less deterioration, at higher temperatures more deterioration over time). Finally, with 10^{-5}M orciprenaline alone curve 1E levels off in a high contractile range. The curve 1E comes very close to the maximal force attainable by the preparation.

The right panel of figure 4.2 gives typical force responses to step changes of frequency. Under control conditions as in 2A, a step increase of frequency from rested state to 1.5 Hz results in a very quick fall of force in 2 to 4 beats, followed by a slow rise toward the steady state. For reduction of frequency from 1.5 Hz to 0.25 Hz force rises in one beat and then falls slowly to the corresponding low level at 0.25 Hz. As shown in 2B, with the orciprenaline-adenosine interaction the slow rise of force for increase of frequency is essentially absent. The slow accumulation of frequency inotropy is replaced by 3 or 4 low amplitude phases in the force transient.

In figure 4.3 the interaction of adenosine with increased extracellular calcium is compared to the adenosine-orciprenaline interaction. Curves given are representative for 8 similar experiments. Also, the negative inotropic effect of adenosine alone is given for this preparation; curve 2A is for 3 X 10^{-4}M adenosine, curve 2B was made immediately after wash-out of the adenosine.

When the negative inotropic effect of 3 X 10^{-4}M adenosine is antagonized by increasing the bath calcium to 8 mM, the rested state contractions are considerably enhanced in comparison to control conditions (compare 2B and 2C). Superficially, it looks as if frequency inotropy production is supressed under these conditions in comparison to the control frequency-force relationship. However, step frequency changes show that frequency inotropy production is present to nearly the same extent as under control conditions

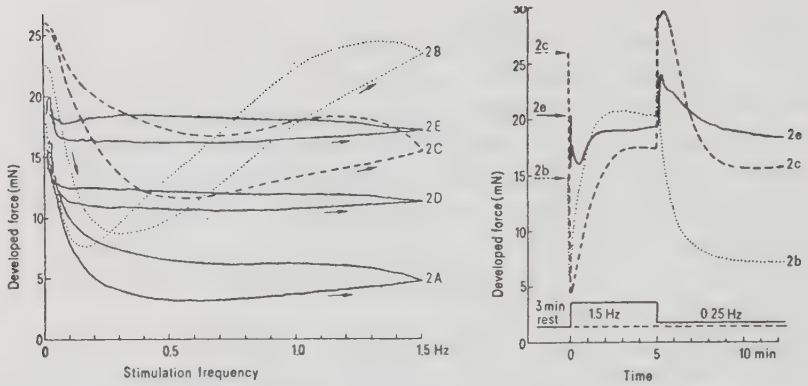

Figure 4.3. All curves from a single atrium. 2A With 3 X 10^{-4}M adenosine alone. 2B Control curve. 2C With 8mM Ca^{++} (final concentration) plus 3 X 10^{-4}M adenosine. 2D 10^{-5}M orciprenaline plus 3 X 10^{-4}M adenosine. 2E 10^{-5}M orciprenaline plus 10^{-4}M adenosine. In the right panel of the figure force responses to step changes of frequency are given in 2b, 2c and 2e for the conditions of 2B, 2C and 2E, respectively. 32°C ; 2.5 mM Ca^{++}. 2B and 2C are retraced.

(compare frequency jumps 2b and 2c). The interaction of adenosine with increased extracellular calcium is only an example of a pseudo-reduction of frequency inotropy production. In frequency-force loops the normal production of frequency inotropy is simply masked by a shift to the **right** of the Kruta part of the relationship. This action of calcium will be dealt with again in chapter 8. The important conclusion here is that an increase of the bath calcium and the negative inotropic effect of adenosine really do antagonize one another on the mechanism of frequency inotropy production.

The curves 2D and 2E give the interaction of adenosine with 10^{-5}M orciprenaline in this preparation. Between curves 2D and 2E the adenosine concentration was reduced from 3 X 10^{-4}M to 10^{-4}M. Note that in these curves the Kruta part of the frequency-force relationship is either masked or reduced. The clear difference in the Kruta parts of these curves and the curve 2C for the calcium-adenosine interaction must reside in different actions of ß-adrenergic stimulation and increasing the extracellular calcium on the mechanism of the Kruta part; adenosine is common to all of the curves.

4.3. Interactions of nifedipine and ß-adrenergic stimulation by orciprenaline.

Figure 4.4 presents representative interactions of nifedipine[§] and orciprenaline on the frequency-force relationship. Curve 1 is a control loop for the atrium. For the curve 2 the positive inotropic effect of 10^{-5}M orciprenaline is antagonized by the negative inotropic effect of 2×10^{-7}M nifedipine. As with the adenosine-orciprenaline combination the frequency-force relationship is greatly flatter than under control conditions. Also, the entire curve shifts downward in a parallel manner when the nifedipine concentration is increased (from curve 2 to curve 3 the nifedipine concentration was increased to 4×10^{-7}M).

Although this interaction is quite similar to the adenosine—orciprenaline interaction, it is distinguishable by two criteria: 1) Frequency-force loops are somewhat wider when orciprenaline is antagonized by nifedipine, and the curve part for increasing frequency has a slight downward tendency in the high frequency range. 2) In step changes of frequency, polyphasic force transients are rare with the nifedipine-orciprenaline combination (see figure 4.5.). For an increase of frequency with the orciprenaline-adenosine combination, contraction force usually rises in a very rapid phase, then decreases more slowly, and finally increases slowly toward steady state. These low amplitude phases usually correspond only to a small, slow phase with the orciprenaline-nifedipine combination.

4.4. Interactions of adenosine and phosphodiesterase inhibition by IBMX.

As demonstrated in figure 4.6, the Bowditch part of the frequency-force relationship is also greatly flatter than under control conditions when the negative inotropic effect of adenosine is antagonized by the positive inotro-

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§ Nifedipine is unstable in light. Therefore, a black cape was placed over the muscle bath for these experiments. Also, nifedipine washes out of muscle preparations only very slowly, so that a randomization of curve order is impossible.

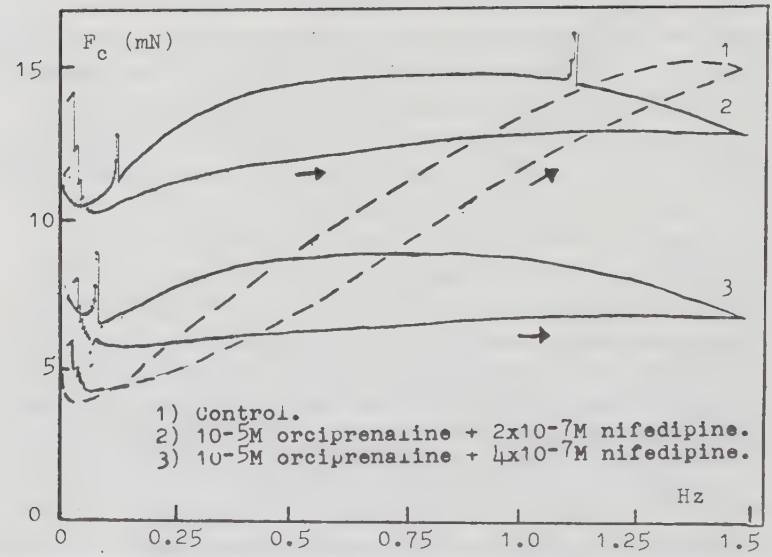


Figure 4.4. Interactions of nifedipine and orciprenaline on the frequency-force relationship. 32°C ; 2.5 mM Ca^{++}. Curve 1 was retraced.

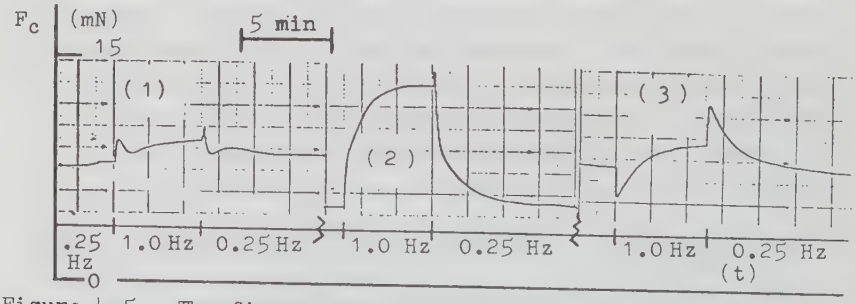


Figure 4.5. The figure compares force transients for step changes of frequency from 0.25 to 1.0 Hz for 5 min and back to 0.25 Hz on a single preparation. 1) 10-5M orciprenaline + 10-5M adenosine (+ 10-7g/ml dipyridamole). 2) control transients after drug wash-out. 3) 10-5M orciprenaline + 2x10-7M nifedipine.

No consistent differences between effects of large concentrations of adenosine or smaller concentrations of adenosine in combination with 10-7g/ml dipyridamole were found in frequency-force studies or responses to step changes of frequency.

The basic differences between the frequency jump 1 and frequency jump 3 were confirmed in 6 similar experiments.

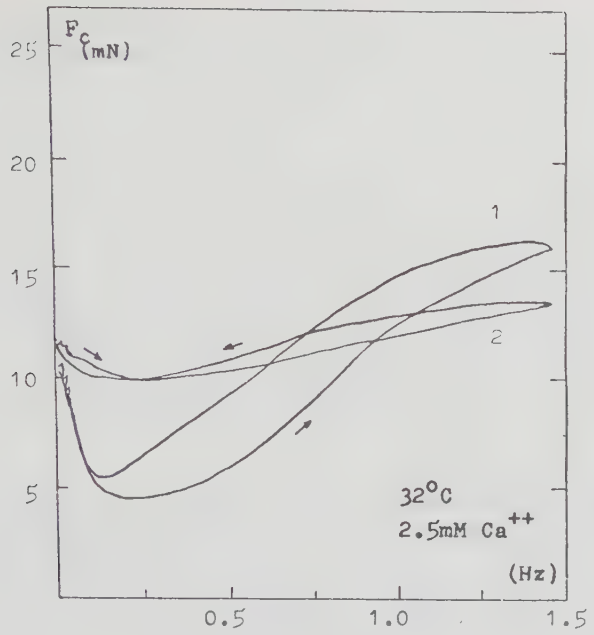


Figure 4.6. Interaction of IBMX and adenosine on the frequency-force relationship. Curve 1, control. Curve 2, 10^{-4}M IBMX + 2 X 10^{-4}M adenosine. (for 1 and 2; 2 X 10^{-7}M dipyridamole). Representative for 6 similar experiments.

pic effect of IBMX. For these experiments 2 X 10^{-7}g/ml dipyridamol was present during all curves. Methylxanthines inhibit competetively most adenosine actions, including the negative inotropic effect of adenosine on guinea-pig atrium (Szentmiklośi et al., 1975). Accordingly, higher concentrations of adenosine are needed to antagonize the effect of IBMX than of orciprenaline. To avoid use of extremely high adenosine concentrations, dipyridamole can be added to inhibit adenosine uptake (see Degenring et al.,1976) and thus enhance its extracellular actions. IBMX was selected for these experiments because it has been shown to mimic the effects of ß-adrenergic stimulation with the least "side-effects" in heart from a group of several phosphodiesterase inhibitors. IBMX increases the speed of relaxation and shifts the con-

centration-response relation for adrenergic stimulation strongly to lower concentrations in guinea pig papillary muscle (Korth, 1978). Other unwanted effects of methylxanthines are not pronounced.

The positive inotropic effect of IBMX is not as strong as maximal ß-adrenergic stimulation (no curves presented). Therefore, it is not surprising that a small amount of frequency inotropy still accumulates with the IBMX-adenosine combination. None-the-less, the IBMX-adenosine interaction is clearly quite similar to the orciprenaline-adenosine interaction; the frequency-force relationship is greatly flatter than under control conditions, and polyphasic frequency jumps are common (no curves presented).

4.5. Other interactions studied.

In order to gain a better perspective on the adenosine-orciprenaline interaction, several other negative and positive inotropic agents were substituted for adenosine and orciprenaline, respectively. In the interest of brevity, these results are communicated only briefly.

As expected, the ß-blocker propranolol reverses the effects of ß-adrenergic stimulation so that frequency inotropy accumulates to the same extent as under control conditions. Only one negative inotropic agent, carbachol, was found to at least in part antagonize the effects of ß-adrenergic stimulation, so that there is a sizable increase of contraction force with increase of frequency (curves for this interaction under other conditions are presented in chapter 6). When the effect of nearly maximal ß-adrenergic stimulation was inhibited by carbachol, the slope of the rising frequency-force relationship was significantly less than for control curves ($p<0.01$), but significantly more than for the orciprenaline interaction ($p<0.01$; $n=7$; Student's t-test for paired observations).

Other negative inotropic agents studied in combination with ß-adrenergic stimulation were verapamil, bath calcium reduction, barbiturates, diphenylhydantoine and trifluorperazine. High concentrations of barbiturates or diphenylhydantoine in combination with orciprenaline also result

in the near abolition of frequency inotropy production; polyphasic frequency jumps are very common. Thus, the absense of polyphasic frequency jumps with the nifedipine-orciprenaline combination seems to be an exception rather than the rule. In contrast to the other negative inotropic agents listed above, a reduction of the bath calcium results in a reduction of the rested state contractions. When calcium reduction is used to antagonize the positive inotropic effect of strong ß-adrenergic stimulation, the frequency-force relationship also becomes greatly flatter than under control conditions (some of the effects of calcium reduction will be presented in chapters 6 and 7). The effect of verapamil is very clearly different than the above agents; when the effect of ß-stimulation is antagonized by verapamil, the frequency-force relationship may be very flat, and there never is a rising Bowditich part. However, when the verapamil concentration is increased an inverted frequency-force relationship results; with increase of frequency steady state contraction force decreases markedly. In contrast to the other agents, verapamil does not antagonize the positive inotropic effect of ß-adrenergic stimulation on the rested state contractions. This general picture quite certainly represents the "use-dependence" of this "calcium antagonist" (Bayer et al., 1975). Trifluorperazine was found to share some of the effects of verapamil; an inverted frequency-force relationship results when ß-adrenergic stimulation is antagonized by high concentrations of trifluorperazine.

When the negative inotropic effect of adenosine is antagonized by heart glycosides, the resulting frequency-force relationship is relatively similar to the high-calcium/adenosine relationship. Rested state contractions are very high and the Kruta part of the relationship is shifted out to higher frequencies. Frequency jumps for step increase of frequency sometimes show a very slow positive phase. Reduction of the bath potassium to 1 mM also has similar effects; when this positive inotropic effect is used to antagonize the negative inotropic effect of adenosine, exceedingly wide frequency-force loops result as reflection of a very slowly accumulating positive inotropic effect of activation.

4.6. Discussion.

Koch-Weser and Blinks (1963) criticized a number of papers in which a certain drug or treatment was reported to "abolish" the interval-strength relationship. The effects described in these papers could be explained by a flattening of the interval-strength relationship as determined by the contractile extremes of a preparation. Even a flattening of the relationship at intermediate contractile levels does not necessarily represent a reduction or elimination of any of the underlying mechanisms. Such an example is the interaction of high bath calcium with adenosine; frequency jumps show that the basic negative and positive inotropic effects of activation are operative, although the steady state frequency-force relationship is quite flat.

Under the opposed actions of orciprenaline and adenosine something fundamental to the production of frequency inotropy has indeed been greatly reduced or abolished. Not only is the frequency-force relationship more-or-less a horizontal line at an intermediate contractile level, the slowly developing positive inotropic effect of activation is strongly reduced in frequency jumps. It is logical to conclude that the crucial manipulations in this interaction are a stimulation of the cAMP system and a reduction of the slow inward current. Phosphodiesterase inhibition by IBMX roughly mimics the ß-adrenergic stimulation in the interaction, and nifedipine roughly mimics the effect of adenosine in the interaction. The findings suggest a new approach to get a grip on the cellular mechanism of the PIEA; if we know how to specifically abolish the mechanism of frequency inotropy production in an intermediate contractile range, it should be possible to deduce something about the identity of the PIEA.

The interaction of orciprenaline and adenosine was presented as a contradiction to the hypothesis that frequency inotropy results from an accumulation of calcium in an intracellular store. If the main mechanism of the positive inotropic effect of ß-adrenergic stimulation results from an enhanced slow calcium inward current, then a reduction of the slow calcium inward current by other means should antagonize the effect of ß-adrenergic stimulation on the frequency-force re-

lationship. Might the observations none-the-less be accounted for in terms of the "calcium accumulation" hypothesis by making some additional assumptions to the simplest consideration of this hypothesis?

All possibilities, which I can conceive of, fall far short of being able to deal with the experimental findings: 1) A stimulation of the cAMP system certainly results in more effects than just an increase of the slow inward current. For example, the sarcoplasmic reticulum is probably phosphorylated. Perhaps the phosphorylation of the SR results in its modification so that calcium no longer can accumulate with increase of frequency. This might be possible if calcium loss between beats from the SR were stimulated by ß-adrenergic stimulation. When such a situtation is simulated, however, one finds that the mechanism of frequency inotropy would not be abolished. An increase of slow inward current would shift frequency inotropy accumulation to a lower frequency range; an acceleration of calcium loss from the SR would shift frequency inotropy accumulation to higher frequencies. No reason is given for an abolition of the underlying mechanism. 2) Adenosine and nifedipine could reduce the capacity of the intracellular store for accumulation of calcium and thereby reduce the potential for frequency inotropy production. This idea must be discounted since the effects of these agents on the mechanism of frequency inotropy are antagonized by increasing the bath calcium. The "potential fill level" of the SR is obviously not reduced by these agents. 3) Adenosine and nifedipine might activate a beat-dependent cellular loss of calcium in addition to their reduction of slow inward current. If such an effect would just counteract the effect of ß-adrenergic simulation on calcium accumulation, frequency inotropy accumulation might be abolished. However, in order to explain the parallel, downward shift of the relationship by increasing the adenosine or nifedipine concentration, it would have to be assumed that the effect of these substances on calcium loss becomes less with increasing concentrations in proportion to the effects on slow inward current. To summarize, even with all speculations permitted it does not seem possible to explain the

pharmacological behavior of the interval-strength relationship in terms of the "calcium accumulation hypothesis." It seems imperative to consider other possible mechanisms of frequency inotropy production.

How might the behavior described be accounted for by assuming a cAMP-dependent mechanism of frequency inotropy production, as proposed by Robison et al. (1965) and again by Hilgemann et al. (1977)? This hypothesis assumes that ß-adrenergic stimulation and the PIEA use a common mechanism for producing their positive inotropic effects. The final step of both mechanisms would be the phosphorylation of certain substrates, whose change by phosphorylation results in a greater amount of calcium becoming available for contraction. Since the positive inotropic effect of ß-adrenergic stimulation probably results from an increased slow calcium inward current, the most likely common mechanism would be a phosphorylation of slow calcium channels, resulting in the availability of more channels for opening. Both ß-adrenergic stimulation and increasing the contraction frequency would lead to phosphorylation of slow calcium channels; if nearly maximal ß-adrenergic stimulation leads to a sizeable phosphorylation of slow calcium channels at rest, the normal mechanism of frequency inotropy production would be precluded by this effect. Reduction of slow inward current by cAMP-independent means would antagonize the effect of ß-adrenergic stimulation on contractile strength, but not on the mechanism of frequency inotropy production. The result of these combined actions would be the abolition of frequency inotropy production at an intermediate contractile level.

This hypothesis could only explain the abolition of frequency inotropy production by a stimulation of the cAMP system and a reduction of slow inward current, if the contraction strength in the Bowditch part of the frequency-force relationship were quite directly proportional to the size of the slow inward current. Such a proportionality would be given by either the proposal that calcium release from intracellular stores with each beat is graded by the slow inward

current (Fabiato and Fabiato, 1977) or that the calcium entering the cell with each beat is enough to activate contraction (Langer et al., 1976). If slow inward current were simply priming the intracellular calcium store for release, it would be expected that the fill level of the store would increase and produce frequency inotropy under the conditions where the mechanism of frequency inotropy production has been largely abolished.

The assumption of a proportionality between slow inward current and the strength of contraction cannot, however, deal with all of the effects presented in this chapter. The Kruta part of the frequency-force relationship is quite insensitive to all of the agents which reduce slow inward current (carbachol, adenosine and nifedipine). This finding cannot be explained by proposing a use-dependence of these actions in atrium. When ß-adrenergic stimulation is used to increase the rested state contractions, each of those interventions can reverse its positive inotropic effect (for the effects of adenosine and nifedipine, see figures 4.2 and 4.4). The lack of effect of these substances on the control frequency-force relationships in the lowest frequency range (figure 4.1) must indicate that the slow inward current is not critically related to the activation of the atrial rested state contractions. The hypothesis that slow inward current increases with increase of frequency predicts that the slow inward current at the rested state would be quite small. If this is the case, its further reduction by the above agents would not be expected to exert a marked inotropic effect on the size of the rested state contractions.

Further work is suggested at three levels: 1) If slow inward current increases in atrium with increase of frequency, an electrophysiological correlate to this effect should be identifiable. Unfortunately, no electrophysiological means were available to me for this work. Even if they had been available, a definitive proof or rebuttal of the above hypothesis cannot be expected. Since voltage clamp experiments are problematic on atrial preparations, indirect indicators of the slow inward current would have to be taken (plateau

height, rate of depolarization after inactivation of fast sodium channels). None of these indicators are necessarily reliable. 2) Biochemical work could help to pin down the exact step in the cAMP system which would be responsible for the production of frequency inotropy. 3)Further work at the functional level might better characterize both the mechanism of frequency inotropy and the electromechanical-coupling system in which this effect operates. Points 2 and 3 are the goals of the next three chapters.

4.7 Summary of chapter 4.

1) The normal rested state contractions of guinea pig atrium are largely insensitive to agents which reduce slow inward current.

2) The normal frequency-force relationship is largely abolished by antagonizing the positive inotropic effect of nearly maximal ß-adrenergic stimulation by adenosine.

3) Increasing the bath calcium does not mimic the effect of ß-adrenergic stimulation in this interaction.

4) Nifedipine can for the most part mimic the effect of adenosine in the interaction.

5) IBMX can for the most part mimic the effect of ß-adrenergic stimulation in the interaction.

6) It seems difficult or impossible to explain results in terms of the hypothesis that frequency inotropy produc-in guinea pig atrium simply reflects an intracellular accumulation of calcium in the store from where it is released.

5. Effects of contraction frequency and adenosine on particulate-bound and total cAMP levels.

For a careful consideration of the hypothesis that the cAMP system is involved in the production of frequency inotropy in atrium, it would obviously be important to know as far as possible what changes in the cAMP system take place with changes of contraction frequency. Since adenosine played a key role in findings which led us to consider this hypothesis, it would also be important to know how or if adenosine acts on the atrial cAMP system.

The figure 5.1 depicts the most important aspects of the cAMP system in heart which could give rise to a beat-dependent positive inotropic effect. The most likely end-target of any effect is thought to be an increased phosphorylation of slow calcium channels. The compartmentalization model of the cAMP system is modified slightly from Corbin et al. (1977): The adenylate cyclase, as is well known, is an integral membrane protein. The cAMP-dependent protein kinase is a holoenzyme, consisting of regulatory and catalytic subunits. Corbin et al. showed that considerable portions of the regulatory subunit are bound loosely to the inside of the sarcolemma. When cAMP rises in the cell, the regulatory subunit binds cAMP and the enzyme dissociates. The catalytic subunit then becomes free to phosphorylate

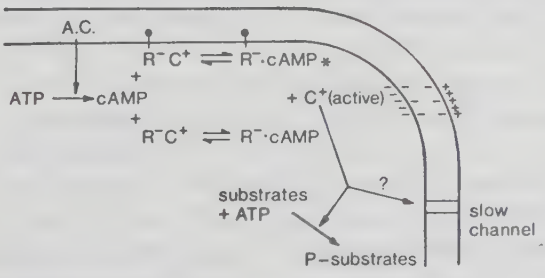


Figure 5.1.

double line = sarcolemma

A.C. = adenylate cyclase

R = regulatory subunit of protein kinase

C = catalytic subunit of protein kinase

Compartmentalization of cAMP and cAMP-dependent protein kinase in heart (modified from Corbin et al., 1977).

Charges have been added to denote the cationic nature of C, the anionic nature of R, the resting membrane potential, and fixed negative charges at the membrane surface.

specific substrates. The regulatory subunit is anionic in nature, the catalytic subunit is strongly cationic in nature, and electrostatic forces are probably important for binding between subunits in the holoenzyme (Corbin et al., 1977b).

A beat-dependent effect (= PIEA) could hypothetically arise in this system at three different levels. 1) The adenylate cyclase might be stimulated by the depolarization. A small amount of cAMP would be produced with each beat, and the cAMP concentration in the cell could increase with increase of frequency. Earlier findings of Brooker (1973) and Wollenberger et al. (1973) lent support to this possibility. These authors reported that the cAMP level in frog heart rises and falls with each contraction. cAMP rose before attainment of peak contraction force and fell before the end of the contraction. Up to now such rapid changes of cAMP levels in mammalian heart have not been reported (Dobson et al., 1976).

2) The dissociation state of the particulate protein kinase could shift in direction of activation with each depolarization, whereby the degree of dissociation of the kinase could increase with increase of frequency. The hypothetical drive for such a mechanism would be an effect on membrane potential changes on the charged protein kinase subunits. Particulate-bound cAMP would accumulate, together with free catalytic subunit, if the rate of protein kinase reassociation were slow.

3) The last step in the reaction chain, the phosphorylation of slow calcium channels, could be facilitated by the depolarization. Either the approach of the positively charged catalytic subunit to the slow calcium channel could depend on depolarization, or phosphorylation sites of the slow channels could be made available for phosphorylation by depolarization. In contrast to the first two possibilities, neither the total cAMP level or the dissociation state of the protein kinase would increase with increase of frequency. Only covalently-bound phosphate would accumulate at the phosphorylation sites of the slow channels.

Corbin et al. (1977) showed that cAMP found in the pellet of heart homogenates is largely or completely associated

with the particulate regulatory subunit of protein kinase. Under carefully controlled homogenization conditions the cAMP found in the pellet is thought to correspond well to in-vivo levels. Therefore, for the present study a method was employed to measure particulate-bound cAMP separately from the remaining cAMP. This allows a look at the state of the cAMP system one step further along the reaction chain between adenylate cyclase and final effect than is possible with conventional cAMP measurements.

Possible effects of contraction frequency on cAMP levels in ventricle have been examined twice. Endoh et al. (1976) reported for rabbit papillary muscle a fall of cAMP levels with increase of frequency. Dobson et al. (1976) reported no change of cAMP with change of frequency in guinea pig papillary muscle. The difference in results could be either due to species variation or to methodological problems in the method of Endoh et al.; 5 to 8 seconds were needed to freeze the tissue samples.

Reported effects of adenosine on cAMP levels in heart are contradictory. Partially isolated adenylate cyclase from heart ventricle of several species is inhibited by adenosine (Bär and McKenzie, 1974). Huang and Drummond (1978) report large increases of cAMP by purinergic stimulation on sliced ventricle and atrium preparations from guinea pigs. Schrader et al. report for intact guinea pig ventricle that adenosine attenuates most effects of catecholamines, probably via an inhibition mechanism at the level of adenylate cyclase. A similar antagonism between adenosine and ß-adrenergic stimulation on the cAMP system and contractility in rat ventricle has been reported by Dobson (1978, 1980). To my knowledge there exists no previous study on the effects of adenosine on cAMP levels in intact atrial preparations. The reports for intact ventricle would indicate that the inotropic effects of adenosine correspond to changes in the cAMP levels; although adenosine does not exert a negative inotropic effect in mammalian ventricle under control conditions, it can attenuate the rise of cAMP and contractility associated with ß-adrenergic stimulation.

In this light it seems important to reexamine Huang and Drummond's findings for guinea pig atrium. The possibility, that adenosine exerts its strong negative inotropic action in atrium via an inhibition of adenylate cyclase and reduction of the cAMP level, is not discounted by the above work.

5.1. Biochemical methods.

Left guinea pig atria (15 to 30 mg) were carefully halved for paired observations. The two parts were mounted on small electrodes in separate muscle baths, which allow Krebs-Henseleit solution to be rapidly recirculated past the muscles by bubbling with 95% O_2/ 5% CO_2. The bath chambers employed in these experiments are normally used for muscle dissection. The electrode holders used were designed so that the muscles would be surrounded by the bath solution flow. Electrical stimuation was set at just above threshhold at 0.2 ms. After equalibriation for 45 min at 0.5 Hz and a chosen treatment, the atria were freeze-clamped in less than 1 s after the last beat and stored in liquid nitrogen.

a) Particulate-bound cAMP was determined with separation conditions as described by Corbin et al (1977): The tissue was pulverized in a stainless steel apparatus at less then -30°C and rapidly homogenized (10 s at 2000 rpm; 3°C) in 0.6 ml of a solution containing 10 mM potassium phosphate, 10 mM EDTA, 0.5 mM IBMX, and 250 mM NaCl at pH 6.8. This procedure was followed immediately by centrifugation at 20000 g for 10 min at 4°C. The supernatant was then removed and the pellet was resuspended in 0.4 ml of the same solution. 50% TCA was added to both fractions to give 6% final concentration, and the samples were vigorously shaken. Protein was determined by the method of Lowry et al. (1951).

cAMP was determined from the two fractions with the protein-binding assay of Gilman (1970). To keep experimental error from additional steps as small as possible, the assay was performed with as few steps as possible. TCA removal by extraction with water-saturated ether (5X with a 10X

greater volume of ether) was found to be adaquate for the assay system, so that chromatography could be left out of the experimental procedure. Dönges et al. (1977) reported no disturbances of the Gilman assay by tissue extracts of guinea pig atria. Also, the assay system is not disturbed by the homogenization solution used for these experiments; the standard cAMP samples for calibration were taken up in the homogenization solution and were subject to the extraction procedure. Four or five cAMP determinations were made from each fraction and values were checked for outlyers by Nalimov's test (Kaiser,1972) with a 95% confidence limit. Total cAMP values given are the sum of particulate and supernatant values related to total tissue protein.

b) cAMP binding protein from tissue samples was determined essentially as described by Corbin et al. (1977): Halved atria were homogenized in 0.4 ml cold homogenization solution as above; whole atria, when used, were homogenized in 0.6 ml. Samples were centrifuged and the particulate fractions were resuspended in 0.3 or 0.5 ml solution, respectively. 20 µl aliquots of the samples were added to 50 µl of a solution containing 50 mM potasium phosphate (pH 6.8), 10 mM EDTA, 0.5 mM IBMX, 0.5 mg/ml histone (Sigma type II-A), 2 M NaCl and 3 µM ^{3}H-cAMP (about 6000 cpm/pmol). Samples were incubated for 90 min at 20°C, and then 1 ml of ice-cold 10 mM potassium phosphate, 1 mM EDTA (pH 6.8) was added. The mixture was filtered on Millipore filters (HA 0.45 um) which were presoaked in the same buffer. The reaction tube was rinsed with 1 ml of the buffer and the filters were washed with 12 ml of the buffer. Filters were dryed at 120°C and radioactivity was counted in a toluene-bases scintilation fluid. Blanks for the reaction mixture without tissue samples were substracted and cAMP binding levels were related to total tissue protein.

c) For statistical analysis Student's t-tests for paired or unpaired observations were applied as appropriate. In all figures the vertical bars represent S.E.M.

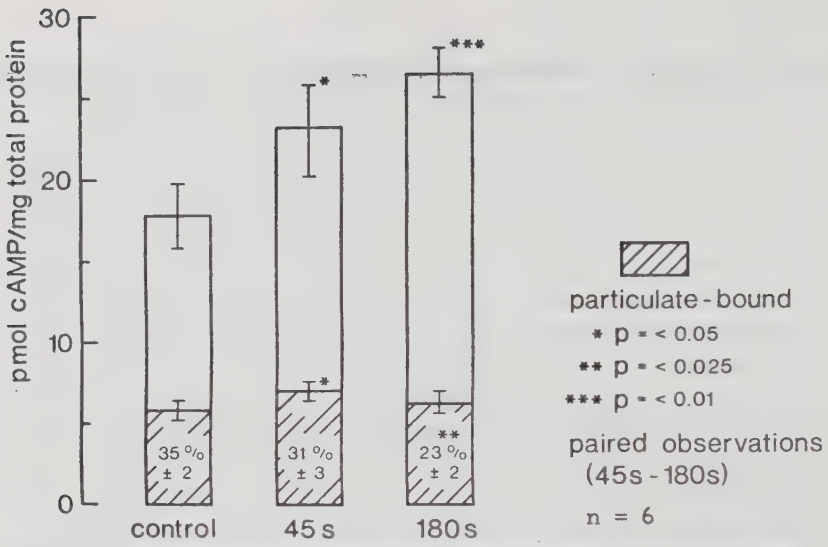


Figure 5.2. The effect of 3 X 10^{-6}M orciprenaline on total and particulate cAMP levels. As in all figures, the lower vertical bars give S.E.M. for particulate cAMP, the upper vertical bars for total cAMP. Numbers in the shaded areas give the percent particulate cAMP, related to total cAMP. 32°C; 2.5 mM calcium; 0.25 Hz.

5.2. Results.

a) Effect of ß-adrenergic stimulation. The figure 5.2 shows the effect of submaximal ß-adrenergic stimulation on particulate and total cAMP levels. For the given control conditions 35% of total cAMP is found associated with the particulate fraction. 3 x 10^{-6}M orciprenaline increases contraction force under these conditions from about 25 to 75% of the preparations' maxima. At 180 s total cAMP is increased by 50% over the control level ($p<0.01$). At 45 s the particulate cAMP level is increased above the control level at just the level of significance ($p=0.05$). Between 45 and 180 s the particulate level tends to decline, so that the increase is no longer significant at 180 s. No simple explanation can be offered for this behavior. It is notable that the increase of particulate cAMP is percentually less than the increase of total cAMP, so that at 180s the per cent particulate cAMP is significantly less than for control conditions.

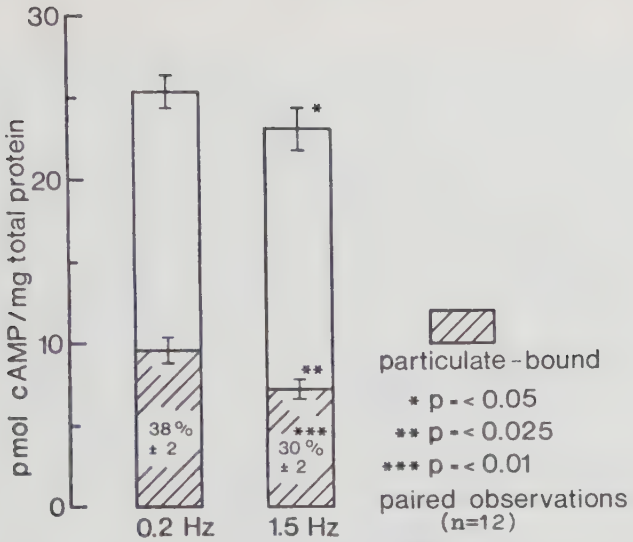


Figure 5.3. The effect of increasing contraction frequency from 0.2 to 1.5 Hz for 3 min on particulate and total cAMP levels. 28°C; 2 mM calcium. See text for further explanations.

b) Effect of contraction frequency. Possible effects of contraction frequency on particulate cAMP were examined under conditions carefully chosen to result in an optimal positive inotropic effect of frequency but still avoiding metabolic fatigue. As mentioned in the introduction, a reduction of the bath temperature enhances frequency inotropy production. Therefore, the experiment in 5.3 was performed at 28°C. Calcium was reduced slightly to 2 mM.

Particulate-bound cAMP decreases with the given increase of frequency by about 30% from the control level ($p<0.025$). Total cAMP decreases just significantly by about the same absolute amount ($p<0.05$), but percentually much less than the particulate cAMP. The percent bound cAMP decreases from 38 to 30% ($p<0.01$). The loss of particulate-bound cAMP does not seem to be explainable by the small fall of total cAMP; the supernatant cAMP does not change at all.

Note that the total and particulate cAMP levels are higher than for the control conditions of figure 5.2. Reinhardt et al.(1978) have shown that temperature reduction increases cAMP levels in guinea pig atria.

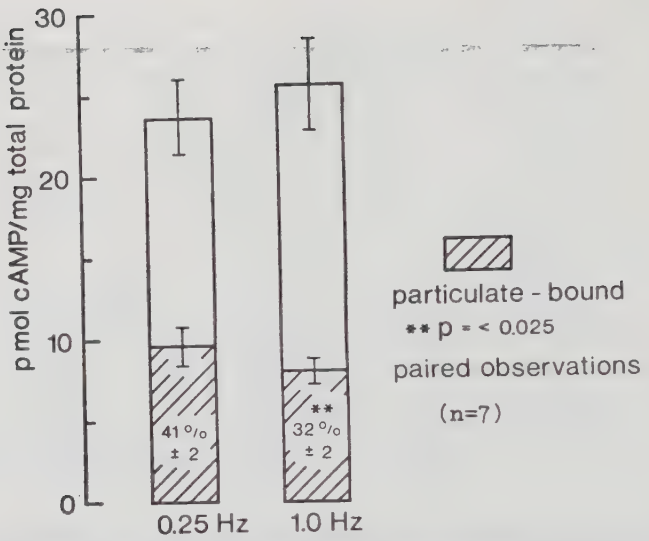


Figure 5.4. The effect of contraction frequency at 32°C with 3 x 10^{-6}M orciprenaline and calcium reduced to 0.8 mM to avoid metabolic fatigue. See text for further explanations.

In the experiment presented in figure 5.4 atria were incubated at 0.5 Hz for 1 h with 3 x 10^{-6}M orciprenaline; calcium was reduced to 0.8 mM to avoid metabolic fatigue. In one atrial half the frequency was reduced to 0.25 Hz, and in the other atrial half the frequency was increased to 1.0 Hz for 4 min. The per cent particulate-bound cAMP is significantly less at 1.0 Hz ($p<0.025$) with clearly no decrease of total cAMP at the higher frequency. The absolute particulate cAMP levels are not reduced at the 95% significance level at 1.0 Hz. None-the-less, the reduction of the per cent particulate bound cAMP must be the result of some factor influencing its binding other than the supernatant cAMP level.

Note: The per cent particulate-bound cAMP is a more sensitive measure than the absolute cAMP levels, since no protein measurements are needed for calculation of the per cent values.

No changes of cAMP levels were found with frequency change when the total cAMP levels were less than in figures 5.3 and 5.4; for example, at 32°C without orciprenaline. Also,

no significant changes were found by increasing the bath calcium from 0.5 to 3.0 mM for 3 min at 0.2 Hz (28°C). Inconclusive results were obtained by depolarizing preparations by replacing 75% of the bath sodium for potassium; under these conditions total cAMP rose significantly in spite of the presense of 2×10^{-5}M propranolol to block the effects of catecholamine release due to depolarization of sympathetic nerve endings.

c) cAMP binding sites. The finding that particulate-bound cAMP decreases with increase of frequency, when total cAMP levels are intermediate to high, could be explained by either a loss of particulate-bound cAMP alone or by a loss of cAMP binding protein as well from membranes. Therefore, an assay for cAMP binding protein in tissue samples was used to examine the possibility that binding protein might be lost from particulate sites with increase of frequency.

The experiment in figure 5.5 was designed to test whether bound cAMP is completely exchanged for unbound cAMP in the assay system. The time course of cAMP binding was followed in particulate and supernatant fractions over time. Half of the samples were incubated with 3 μM cold cAMP for 1 h, and half of the samples were incubated with 3 μM ^{3}H-cAMP from t_o immediately after homogenization and centrifugation. After 1 h radioactivity was added to the cold samples and exchange of the radioactivity was followed. Both particulate and supernatant bound-cAMP clearly exchange completely with radioactive cAMP. Exchange of bound supernatant cAMP is apparantly somewhat slower than bound particulate cAMP.

About 20 pmol/ mg total protein cAMP binding sites are found in guinea pig atria. Three to four times more binding activity is found in the supernatant fraction than in the particulate fraction. Note that binding in the particulate fraction shows a reduction between 30 and 80 min, so that the binding level at 90 min, the standard time used for the assay, may be an underestimation of binding sites. The reason for this loss of binding activity is unknown.

Although only 4.5 pmol/mg protein particulate binding is found in the binding assay at 90 min, up to 11 pmol/mg pro-

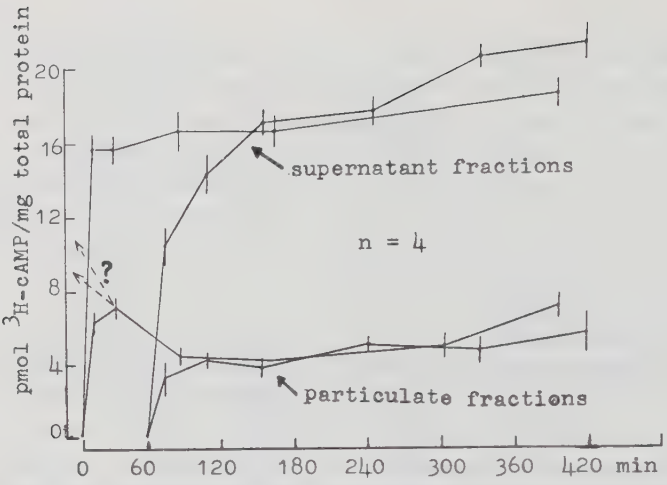


Figure 5.5. The time course of ^{3}H-cAMP binding in particulate and supernatant fractions of homogenates immediately after homogenization. One group of particulate and supernatant samples were incubated for the first 60 min with 3 µM cold cAMP. Radioactivity was then added to follow the exchange of bound for unbound cAMP. Note that binding to the particulate fraction decreases between the first 30 and 80 min of incubation. See text for further explanations.

tein particulate-bound cAMP have been found in tissue samples using the Gilman assay. This fairly large discrepancy may be explained in part by the loss of binding activity in the particulate fractions over time in the binding assay. It might as well be explained by a trapping of cAMP binding protein in vescicles formed at homogenization. Terasaki and Brooker (1977) reported a similar discrepancy in work with particulate-bound cAMP in rat atria. Corbin et al. (1977) reported less particulate-bound cAMP in ventricular homogenates than binding sites found by the protein binding assay.

1 mM adenosine has no effect on the amount of cAMP binding found in either the particulate or supernatant fractions or the time course of binding. This eliminates the possibility that part of binding could be due to binding to purinergic receptors or other adenosine-binding proteins.

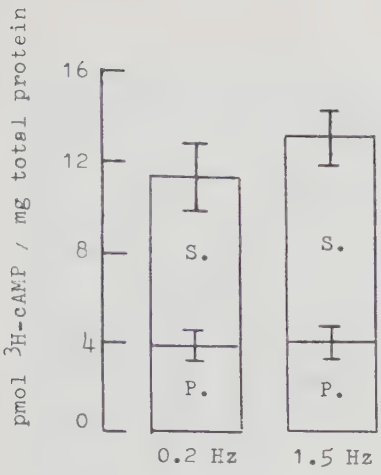


Figure 5.6. The figure demonstrates the lack of effect of contraction frequency on particulate cAMP binding sites. The upper vertical bars correspond to total cAMP-binding (S.E.M), the lower bars correspond to particulate cAMP binding. P.= particulate. S.= supernatant. n = 5.

The figure 5.6 shows the lack of effect of an increase of frequency from 0.2 to 1.5 Hz at 28°C for 3 min on the particulate cAMP binding sites. The values given correspond to binding in the assay system at 90 min. Since there is no loss of particulate binding sites with increase of frequency, the loss of particulate cAMP with increase of frequency must reflect a loss of the bound cAMP alone. Since changes of the total cAMP level do not seem to account for the effect, it is concluded that the affinity of the particulate cAMP receptors for cAMP is reduced by increasing contraction frequency.

d) Effects of adenosine on cAMP levels. In the series presented in figure 5.7 (A) total cAMP levels were measured in response to adenosine at 1 Hz, administered 2 min prior to freezing. 10^{-5}M adenosine (= 30% negative inotropic effect) showed no effect, and 10^{-4}M adenosine (= 80% negative inotropic effect) increased the total cAMP level by nearly 80% ($p<0.05$). In the series presented in 5.7 (B) the atria were pretreated with 10^{-7} g/ml dipyridamole, so that for equivalently strong negative inotropic effects about a factor 10 less adenosine can be used. For the B-series both particulate and total cAMP values were measured. Other conditions were as in the A-series. One group of atria received no treatment for 1 h; a second group received 10^{-7}

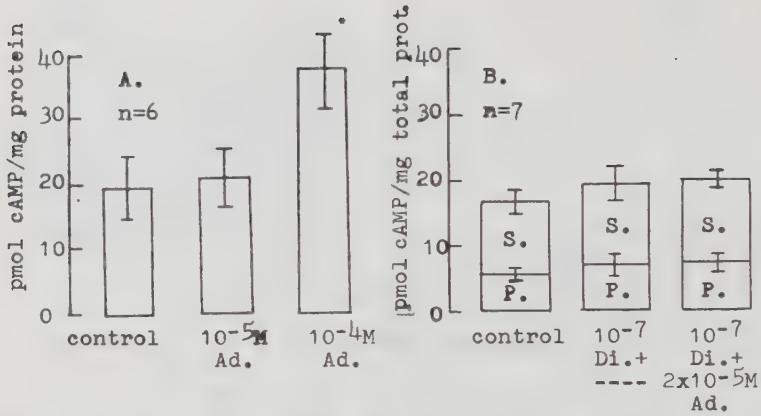


Figure 5.7. The figure shows the effect of adenosine (=Ad.) on cAMP levels with and without dipyridamole (Di.) in concentration of 10^{-7}g/ml. With the dipyridamol pretreatment the negative inotropic effect of $2x10^{-5}$M adenosine is at least as strong as 10^{-4}M adenosine alone. 32°C; 2.5 mM Ca^{++}; 1 Hz. The star in A indicates $p<0.05$. For experiments with dipyridamole particulate and supernatant cAMP levels were measured separately. See text for further explanations.

g/ml dipyridamole for 1 h; a third group received 10^{-7}g/ml dipyridamole for 1 h followed by 2 X 10^{-5}M adenosine for 2 min. Under these conditions the negative inotropic effect of adenosine is not accompanied by an increase of cAMP as in figure 5.7 (A). It may be concluded from these experiments that adenosine can increase the cAMP level of beating atria, but this effect has no relation to the negative inotropic effect.

5.3. Discussion.

The cAMP levels found in this study for left guinea pig atria are among the highest known to me in the cAMP literature for heart preparations. There are probably several reasons for this. First, a survey of the cAMP literature for heart shows that cAMP values found for atrial preparations are consistently higher than those found for ventricular preparations. Second, in the choice of experimental conditions in this study great care was taken to insure that oxygenation of preparations was as good as possible

and that preparations were not metabolically stressed by the various inotropic interventions. Since the cAMP system is dependent on ATP, a better metabolic state would probably favor higher cAMP levels. Third, to yield thin muscle preparations, the animals used for these experiments were younger than those used in other studies. Age could as well be a factor of importance. Fourth, the possibility cannot be completely eliminated that the cAMP values measured here are somewhat inflated do to disturbances in the atrium homogenates.

If we assume that 50% of all cAMP is bound to particulate and soluble protein kinase, the free concentration of cAMP in the cell would still be more than 1 μM. It is well-known that isolated cAMP protein kinases are fully activated at 1 μM, so that there would be no room for control of kinase activity by further increases of the cAMP concentration. This contradiction has come up several times in the literature. It is now established that the apparent K_a for protein kinase activity increases with enzyme concentration; with the higher concentrations of protein kinase in the cell the concentration of cAMP would also have to be higher to regulate the enzyme (see Nimmo and Cohen, 1977). As expected from this consideration, the amount of cAMP binding found in homogenates of guinea pig atria in this study is 2 or 3 times greater than levels described for various ventricular preparations (Corbin et al., 1977). Could this be an indication that cAMP kinase substrates are also present in higher concentrations in guinea pig atrium?

The present work clearly eliminates the possibilities that either total or particulate cAMP accumulates with increase of frequency in guinea pig atrium. If a cAMP-dependent mechanism of frequency inotropy production exists, it must result in a beat-dependent phosphorylation of some cAMP kinase substrates without involving an increase of total cAMP or an increased dissociation of particulate protein kinase with increase of frequency. Of the three possibilities given in the introduction to

this chapter, the idea that the final phosphorylation step in the cAMP system could be beat-dependent is favored as a working hypothesis for further consideration.

A clue about the nature of such a mechanism can be taken from the finding that particulate-bound cAMP tends to decrease with increase of frequency. According to the scheme in figure 4.1, a loss of particulate cAMP would be accompanied by a return of catalytic subunit to the regulatory subunit bound on the inside of the membrane. The loss of particulate cAMP could be explained by a general dependence of the approach of the catalytic subunit to the inside of the membrane on frequency of contraction. The measured loss of particulate cAMP would reflect a larger effect; if the catalytic subunit wins a better approach to the membrane at depolarization, it would tend to accumulate at the inside of the membrane as frequency is increased. The result would be first a release of some cAMP from the membrane-bound regulatory subunit, and second a beat-dependent phosphorylation of slow calcium channels. This interpretation is admittedly quite speculative in the context of only these experiments. **However, it will be shown in chapters 6 and 8 that this interpretation indeed provides a good basis for explaining a number of findings on the pharmacological behavior of frequency inotropy production which cannot be explained by other available ideas.**

The finding that adenosine does not lower the atrial cAMP level, sometimes even increases it, clearly eliminates the possibility that adenosine might exert its negative inotropic action in atrium via an inhibition of adenylate cyclase. A direct effect of adenosine on potassium permeability (Hartzell, 1979), resulting in a strong shortening of the atrial action potential (Johnson, 1956), and thereby a reduction of slow calcium inward current, seems to be the most likely mechanism. A more direct depressant action on the slow channels cannot be ruled out.

The increase of cAMP found with high adenosine concentrations is quite certainly not related to the negative inotropic effect. With dipyridamole this effect could be dis-

sociated from the negative inotropic effect. An increase by adenosine of the cAMP level, in addition to its negative inotropic effect, could contribute to the abolition of frequency inotropy found with the adenosine-orciprenaline combination. The cAMP level with the combination would be enhanced more than with orciprenaline alone. Phosphorylation at rest would be strengthened, and in this way the preclusion of the mechanism of frequency inotropy would be especially complete. This might as well explain part of the differences between the actions of nifedipine and adenosine as antagonists to ß-adrenergic stimulation.

5.4. Summary of chapter 5.

1) An increase of contraction frequency favors a redistribution of cAMP in guinea pig atrium from a particulate-bound form to the non-particulate fraction.
2) This effect cannot be explained by a reduction of total-cAMP.
3) This effect cannot be explained by a loss of particulate-bound cAMP-binding protein.
4) This effect cannot be explained by an indirect effect of changing contraction strength per se.
5) The effect of frequency on particulate-bound cAMP could be explained by a translocation of free catalytic subunit of cAMP protein kinase to the inside of the sarcolemma with increase of frequency.
6) 10^{-4}M adenosine increases total cAMP in beating atria.
7) The effect of adenosine on total cAMP can be dissociated from its negative inotropic effect.

6. A "real" maximum of frequency inotropy production.

6.1. Introduction.

If there exists a cAMP-dependent mechanism of frequency inotropy production, the most likely mechanism--as outlined in the last chapter--would be a beat-dependent phosphorylation of slow calcium channels by the catalytic subunit of cAMP-dependent protein kinase. It might be envisioned as a collision-mechanism; the depolarization of the membrane or some effect of depolarization would favor the collision of catalytic subunits with slow calcium channels, resulting in the phosphorylation of unphosphorylated channels. The number of channels phosphorylated per beat would depend on a number of factors; 1) the activity of the protein kinase, 2) on the degree to which the probability of phosphorylation is increased by depolarization, 3) on the duration of depolarization in the critical potential range, and 4) on the number of channels already phosphorylated before the beat. Assuming that dephosphorylation is a simple monomolecular process taking place continuously, this hypothesis predicts a hyperbolic relationship between frequency and phosphorylated slow channels (= activatable channels). If contraction force is quite directly related to the number of activatable slow channels in the Bowditch part of the frequency-force relationship, then this curve should as well show a hyperbolic nature. That is to say, the relationship should tend to increase toward a maximum, determined by the number of channels available for phosphorylation and whatever factors determine the translation of phosphorylated channels into contraction force (see figure 6.1).

According to this idea a stimulation of the cAMP system would shift the accumulation of frequency inotropy to lower frequencies. If the slow channels are blocked or the slow inward current reduced by cAMP-independent means, the maximum of the frequency inotropy accumulation should be reduced without a shift of the frequency range in which accumulation takes place. If ß-adrenergic stimulation were antagonized by a cAMP-independent negative inotropic agent, frequency inotropy would accumulate in a low frequency range,

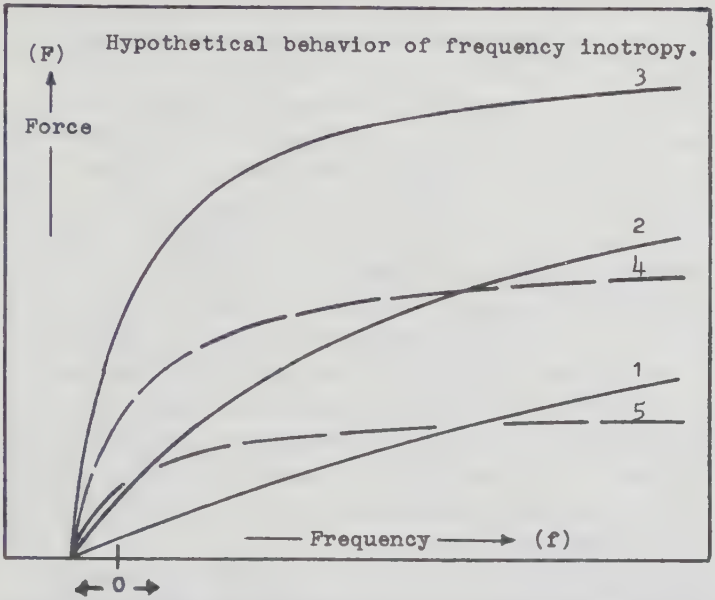


Figure 6.1. The hypothesis that the main mechanism of frequency inotropy production is a beat-dependent phosphorylation of slow calcium channels by cAMP-dependent protein kinase predicts a hyperbolic relationship between frequency and contraction force. The simplest possible situation would be given by the following assumptions: 1) The probability of slow calcium channels being phosphorylated is increased during each beat. 2) Dephosphorylation is a continuous, exponential process. 3) The fraction of phosphorylated slow channels is directly related to the strength of contraction.

$$\text{Contraction Force} = F_{max} \cdot \frac{K_r + F_{PIEA} \cdot f}{K_r + F_{PIEA} \cdot f + K_{PIEA}}$$

F_{max} = contraction force developed at 100% phosphorylation of slow channels.
K_r = rate constant for phosphorylation at rest.
F_{PIEA} = fraction of slow channels phosphorylated with each beat.
f = frequency of contraction
K_{PIEA} = rate constant for dephosphorylation.

Stimulation of the cAMP system would shift the accumulation of frequency inotropy to a lower frequency range (increase of K_r and F_{PIEA} in parallel; curves 1 to 2 to 3). Reduction of slow inward current by cAMP independent means would simply shift the relationship downward with no change of the frequency range of accumulation (reduction of F_{max}).

but the maximum would be reduced. Above a low frequency range the essential mechanism of frequency inotropy production would be exhausted. There would be no further increase of force with increase of frequency unless some other mechanism were operative. Such a behavior would contradict both the hypothesis that the main mechanism of frequency inotropy is an intracellular accumulation of calcium in the store from where it is released and the hypothesis that the main mechanism of frequency inotropy is an accumulation of sodium coupled to a positive inotropic effect. For these hypotheses the degree of phosphorylation of slow channels would be irrelevant; the driving mechanisms would be the amount of calcium or sodium entering the cell per beat. There is no reason to think that either of these factors would be changed by a stimulation of the cAMP system and a reduction of the slow inward current by cAMP-independent means.

To test the predicted behavior it would be necessary to examine the frequency-force relationship under conditions where the Kruta part of the curve is suppressed. Otherwise, it is impossible to accurately judge the behavior of frequency inotropy accumulation in the low frequency range of interest. Furthermore, it would be important use experimental conditions under which strong ß-adrenergic stimulation does not result in maximal force development. In this way a maximum of frequency inotropy production could be differentiated from a maximum of force development as determined by the maximal force which a preparation can generate. Finally, a maximum of frequency inotropy production should be differentiatable from a maximum due to the negative inotropic effect of metabolic fatigue at high frequencies and high contractile levels.

6.2. Suppression of rested state contractions by temperature reduction and "slow-response" conditions.

Three methods of effectively reducing the atrial rested state contractions are known to me. The inhibition of rested state contractions by ryanodine was presented in figure 4.1. In figure 6.2 two further interventions are

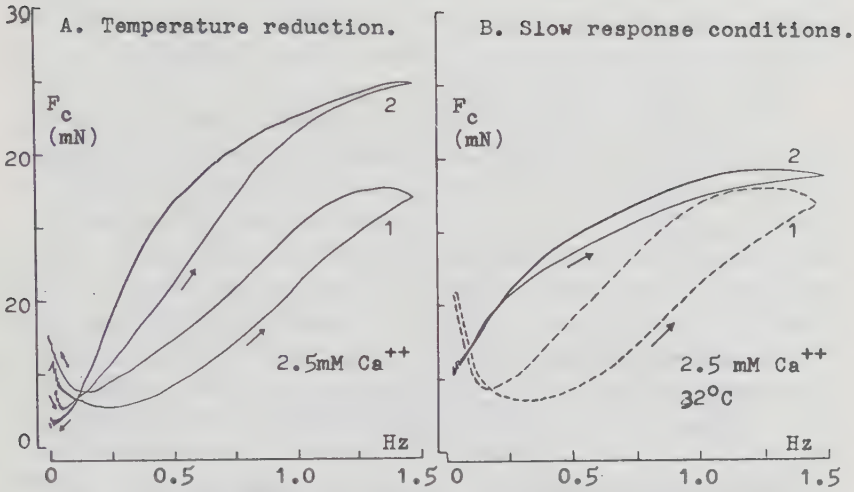


Figure 6.2. Two ways of reducing rested state contractions and thus isolating the Bowditch part of the frequency-force relationship. In the left panel (A) the typical effect of lowering the bath temperature from 32° (1) to 28°C (2) is shown. In the right panel (B) curve 1 is a control and curve 2 is under slow response conditions (10^{-5}M orciprenaline, 12 mM potassium, 2 X 10^{-6}M TTX).

presented which effectively result in suppression of rested state contractions; temperature reduction and slow-response conditions[§]. Under each of these three conditions the hyperbolic nature of frequency inotropy accumulation can be demonstrated, and specific effects on the maximum of the relationship and the frequency range of accumulation can be differentiated. One of the problems of differentiating a maximum of the mechanism of frequency inotropy is apparent in figure 6.2: The curves 2 in each figure seem to be approaching a maximum. In panel A the curve 2 at 28°C could be bent from linearity by the non-linear relationship between force and calcium in the highest force range (see chapter 7.2). In curve 2 in panel B, however, the

[§]In heart muscle, action potentials can be elicited by strong electrical impulses even after blockade of fast sodium channels (= TTX) or their inactivation by depolarization with increased potassium concentrations. Such action potentials are thought to be activated and propagated via the slow calcium channels ("slow responses").

frequency-force relationship is clearly bent toward a maximum in the same force range where the control curve (1) shows a linear increase of force with increase of frequency. The hyperbolic appearance of curve 2 in panel B might reflect the nature of the process underlying accumulation of frequency inotropy.

Of the three possibilities for studying frequency-force relationships with reduced rested state contractions, temperature reduction proved to be most useful for further work. A stimulation of the cAMP system seems to be a prerequisite for slow responses, so that a detailed study of effects of ß-adrenergic stimulation or phosphodiesterase inhibition is difficult under slow response conditions. Also, some preparations were unstable under these conditions. Increasing the potassium concentration (= depolarization) very probably has a number of effects not related to inactivation of sodium channels. Also, the high concentrations of TTX needed to inactivate sodium channels in heart (>10^{-5}M) have effects not unsimilar to those of nifedipine in this preparation (unpublished observations). I have obtained the most stable, reproducible results by a combination of modest depolarization with 12 mM potassium (final concentration) and smaller concentrations of TTX (curve 2 in panel B of figure 6.2). These two interventions inhibit fast sodium conductance in an over-additive way (Baer et al., 1976).

As will be described in 6.6. and 7.4.4., ryanodine seems to create a new phase of frequency inotropy accumulation and decay. After ryanodine treatment the positive inotropic effects of ß-adrenergic stimulation, increasing the bath calcium, and increasing frequency are all reduced in the low frequency range. For these reasons use of ryanodine to analyze the normal mechanism of frequency inotropy production is problematic.

A reduction of bath temperature in fact results in a greater stability of preparations. Control frequency-force relationships have been found to be stable within 10 to 20% over 8 hours at 28°C. The effects of ß-adrenergic and parasympathetic stimulation are enhanced

at lower temperatures. The basic mechanisms of the interval-strength relationship are not fundamentally altered by temperature reduction (to be described in chapters 7 and 8). These (unpublished) observations make temperature reduction a quite ideal intervention for isolated study of the mechanism of frequency inotropy production. In order to keep contractility below the preparations' contractile ceiling, even with ß-adrenergic stimulation, the bath calcium is reduced from 2.5 to 1.2 mM§. This has the effect of reducing the rested state contractions still further than in figure 6.2. If atria are pretreated with strong ß-adrenergic stimulation, the resulting frequency-force relationships after wash-out at 28°C/1.2 mM calcium are stable and show virtually no rested state contractility in frequency-force loops. These are the conditions of experiments presented in 6.3, 6.4 and 6.7.

6.3. Interactions of nifedipine and carbachol with orciprenaline at 28°C, 1.2 mM Ca^{++}.

Figure 6.3 presents typical effects of ß-adrenergic stimulation by orciprenaline in curves 1 to 4; curve 1 is a control relationship, curves 2, 3 and 4 are with 10^{-7}, $4x10^{-7}$ and $1.6x10^{-6}$M orciprenaline respectively. The relationship is shifted to lower frequencies, and the rested state contractions are increased up to about 40% of the preparations' maxima. The highest contractile level reached in curve 4 is not yet the maximum of the preparation. Note that curve 3 shows the beginning signs of metabolic fatigue; the returning part of the curve falls below the rising part of the frequency-force loop. Therefore, it is important to reverse frequency change at a lower frequency for relationships at high contractile levels (curve 4). Note that the rising and falling parts of loop 4 show hysteresis. This as well is taken as a sign that the lack of hysteresis in loop 3 is due

§ In retrospect, 1.0 mM Ca^{++} would have been a better choice. In some experiments the positive inotropic effect of strong ß-adrenergic stimulation clearly brought contraction force very close to the contractile ceiling of preparations.

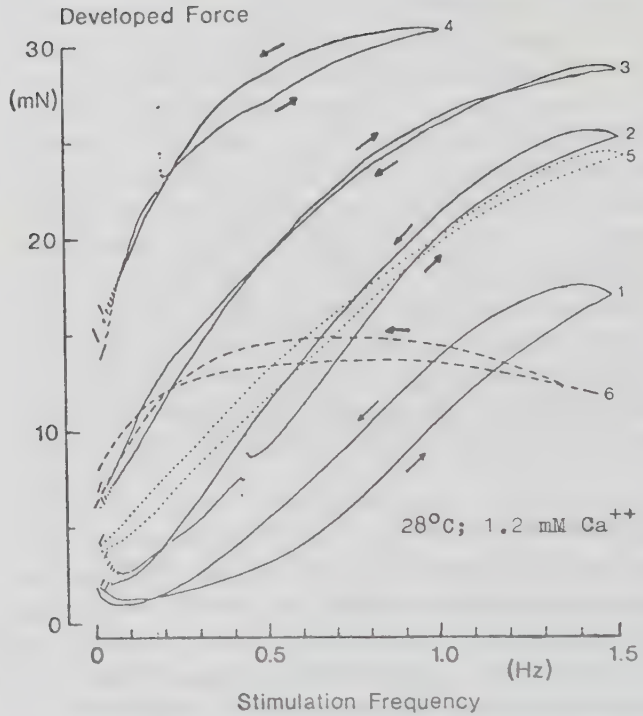


Figure 6.3. The figure demonstrates the "hyperbolic" nature of frequency inotropy production. For experiments at 28°C the rate of automatic frequency change is set somewhat slower (t_{50}, ap. 4 min) than for experiments at 32°C; the decay rate of frequency inotropy is slowed by temperature reduction. Frequency-force loops are for the conditions:

curve 1; control
curve 2; 10^{-7}M orciprenaline
curve 3; 4 x 10^{-7}M orciprenaline
curve 4; 1.6 x 10^{-6}M orciprenaline
curve 5; " " " " " " + 6 x 10^{-8}M carbachol
curve 6; " " " " " " + 5 x 10^{-7}M nifedipine.

Between loops 5 and 6 carbachol was removed pharmacologically by application of 10^{-7}M atropine; a loop made before application of nifedipine (not shown) was practically identical to loop 4. See text for further explanations.

Curves 5 and 6 were retraced for clarity.

metabolic fatigue.

From curve 4 to curve 5, 6 x 10^{-8}M carbachol was added; the similarity between curve 2 and curve 5 demonstrates that under these conditions carbachol can quite "specifically" antagonize the effects of ß-adrenergic stimulation on the frequency-force relationship. Whereas ß-adrenergic stimulation shifts the relationship to lower frequencies, carbachol shifts the relationship back to higher frequencies. After curve 5, 10^{-7}M atropine was added to antagonize the effect of carbachol, and the next curve (not given) was nearly identical to curve 4.

From curve 4 to curve 6, the effect of 5 x 10^{-7}M nifedipine is shown; the curve 6 cuts through curves 1, 2, 3 and 5. The effect of ß-adrenergic stimulation on the frequency range in which frequency inotropy accumulates is left completely unaffected by nifedipine. The frequency-force relationship for curve 4 is simply shifted to a lower contractile range. After nifedipine the frequency-force relationships have a tendency to reach a maximum and then decrease slightly in the highest frequency range, even if the level of contractility of the loop is intermediate or small (see curve 6); this may reflect a certain degree of use-dependence of the nifedipine effect. Such an effect cannot, however, explain the difference between the negative inotropic effects of carbachol and nifedipine under these conditions. Less nifedipine simply reduces the maximum of frequency inotropy production to a lesser extent, more nifedipine to a greater extent (no curves presented). A clear deviation of the "real" behavior from "ideal" behavior in these curves is that the control loops begin with a bowing in the low frequency range; this is very probably a reflection of the non-linear relationship between calcium and force development in the lowest force range.

6.4. Interactions of ATP and carbachol with orciprenaline at 28°C, 1.2 mM Ca^{++}.

Representative interactions of purinergic stimulation (ATP) and cholinergic stimulation (carbachol) with ß-adrenergic stimulation (orciprenaline) at 28°C/1.2 mM calcium are shown in figure 6.4. For this experimental series

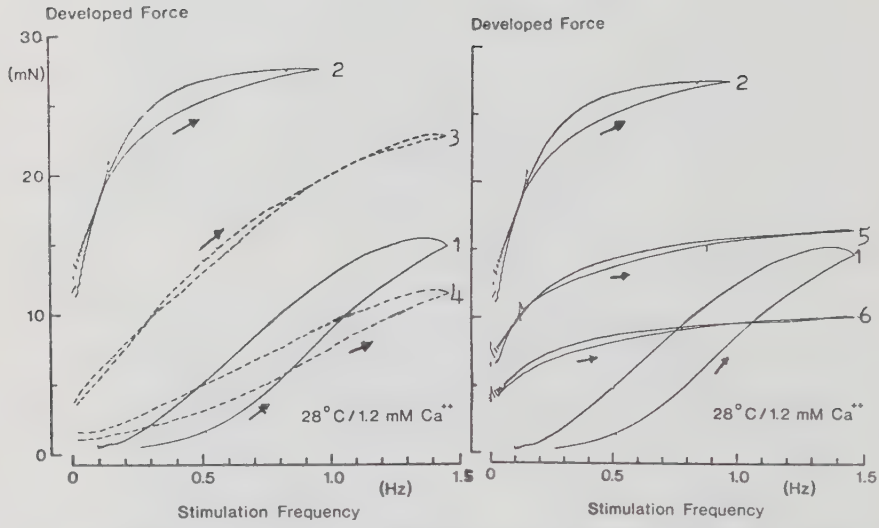


Figure 6.4. Interactions of carbachol and ATP with orciprenaline at 28°C/1.2 mM calcium. The figure is divided into two parts for clarity.

curve 1; control
curve 2; 1.6 x 10^{-6}M orciprenaline
curve 3; " " " " " + 6 x 10^{-8}M carbachol
curve 4; " " " " " + 1.2 x 10^{-7}M "
curve 5; " " " " " + 3 x 10^{-5}M ATP
curve 6; " " " " " + 10^{-4}M "

Curves were made in the following order; 1,6,5,4,3,2.

Between curves drugs were washed-out and readded in the given concentrations. Curves 3 and 4 were retraced as dashed lines for clarity.

ATP was substituted for adenosine as purinergic stimulant. This excludes the possibility that any of the effects seen could be due to an intracellular action; ATP is impermeable. Also, it excludes the possibility that any effects could be due to an adenosine gradient across the muscle preparation; since adenosine is taken up by the cells, the concentration of adenosine at the inner-most cells would probably be less than the bath concentration.

As demonstrated in the left panel of figure 6.4, the carbachol-orciprenaline antagonism on the frequency-force relationship is not perfect. With the carbachol concentration chosen high enough to antagonize the effect of quite strong ß-adrenergic stimulation (curve 4; 1.2×10^{-7}M carbachol + 1.4×10^{-6}M orciprenaline), the frequency-force loop still cuts through the control loop (curve 1). The flattening of the frequency-force relationship for the carbachol-orciprenaline combination in comparison to control is typical for this interaction on over 20 preparations. There are two components to the carbachol effect, one which "specifically" antagonizes the effect of ß-adrenergic stimulation, and one which is adenosine-like. If ß-adrenergic stimulation is still stronger than in the figure 6.4, the adenosine-like effect is more pronounced, and the difference between the effects of cholinergic and purinergic stimulation becomes less prominent (no curves presented).

In the right panel of the figure, the effect of purinergic stimulation on the maximum of the frequency-force relationship under these conditions is presented. After ß-adrenergic stimulation with shift of frequency inotropy accumulation into the low frequency range, the effect of ATP (or adenosine) is simply to shift the relationship downward with no effect on the frequency range of accumulation (curves 2 to 5, and 5 to 6). Note that the relationship shows no tendency to decline in the high frequency range. In this respect the negative inotropic effect of purinergic stimulation is "cleaner" than the effect of nifedipine as presented in figure 6.3.

6.5. Effect of bath calcium and interaction of low bath calcium with orciprenaline..

Inotropic effects of changing the bath calcium are more complex than other inotropic interventions presented thus far. The typical effects of the bath calcium concentration on the frequency-force relationship at 28°C are shown in figure 6.5. The Bowditch part of the relationship appears to be changed in a way not unsimilar to the effect of ß-adrenergic stimulation. The slope of the rising curve is

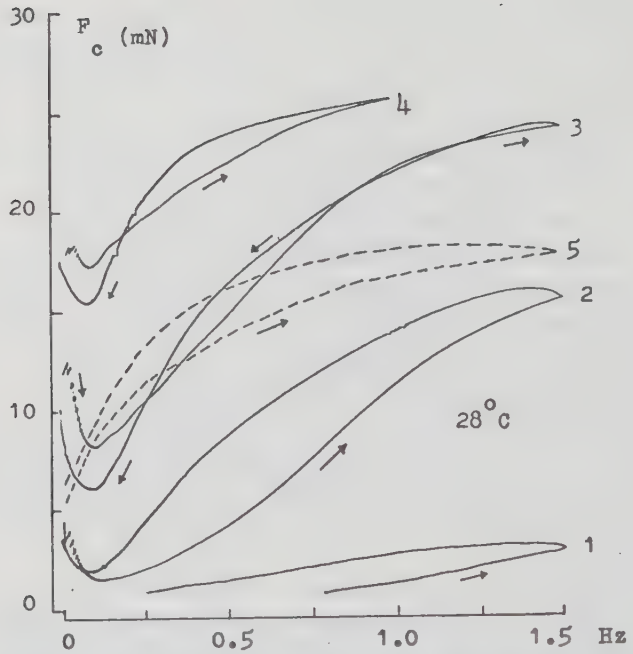


Figure 6.5. The typical effects of changing the bath calcium on the frequency-force relationship at 28°C. Curve 1; 0.7 mM calcium. Curve 2; 2 mM calcium. Curve 3; 4 mM calcium. Curve 4; 8 mM calcium. Curve 5; 0.7 mM calcium plus 10^{-5}M orciprenaline. Curves were made in the following order; 4, 3, 2, 1, 5. Curve 5 was retraced for clarity.

increased and the relationship is shifted to a lower frequency range. In strong contrast to the effect of ß-adrenergic stimulation, increasing the bath calcium enhances the rested state contractions, so that the Kruta part of the relationship reappears at 28°C. Note that in curves 3 and 4 the returning parts of loops droop down below the parts for increase of frequency; this is very likely an after-effect of metabolic stressing at the high frequencies.

For curve 5 in the figure, the negative inotropic effect of reduction of the bath calcium to 0.7 mM was antagonized by 10^{-5}M orciprenaline. This loop cuts through the curve 3 and approaches an intermediate maximum. The curve is bowed in same force range where the curve at 2 mM calcium (curve 2) is quite linear. Thus, the effect of reducing

the bath calcium antagonizes the effect of ß-adrenergic stimulation similarly to purinergic stimulation or nifedipine. Ideally for this classification of the effect of calcium on the Bowditch part of the frequency-force relationship, the curves 3 and 4 in the figure should show a bowing toward a maximum in the same frequency range where the curve 2 begins to show a bow. Obviously, the curves 3 and 4 bow from linearity at lower frequecies than the curve 2; this may be explained by the effects of metabolic fatigue and the non-linear relationship between force and calcium in the high contractile range. One final observation is noteworthy: After reduction of the bath calcium, higher concentrations of orciprenaline are needed to shift the relationship to the left than after either nifedipine or adenosine (or ATP). This could reflect a dampening effect of calcium reduction on some process in the cAMP system.

6.6. Differentiation of different negative inotropic types after ryanodine treatment plus orciprenaline.

After ryanodine treatment (2×10^{-7}M for 20 min, followed by wash-out and continuous application of 3×10^{-9}M) as in panel D of figure 4.1, the Bowditch part of the frequency-force loops shows a normal degree of hysteresis. That is, frequency inotropy accumulates and decays with a half-life about twice as fast as rate of frequency change. As shown in figure 6.6, a subsequent, strong ß-adrenergic stimulation results in a frequency-force loop which shows no hysteresis (curve 1). Frequency inotropy accumulates and decays under these conditions in just a few beats. Although the slow mechanism of frequency inotropy production is eclipsed by the ß-adrenergic stimulation, an apparently different mechanism of frequency inotropy under these conditions still results in a very strong increase of force with increase of frequency. The fact, that the loop part in curve 1 for reduction of frequency falls below the curve part for increase of frequency, probably reflects a small degree of metabolic fatigue. Note as well that an extrasystolic beat in the rising curve part shows

an enhanced contraction strength, not the usual loss of contractility. Although contractile behavior under these conditions (3 x 10^{-9}M ryanodine, 10^{-5}M orciprenaline and 4 mM calcium) is clearly different than for low temperature, adenosine still reduces the "real" maximum of frequency inotropy (curve 1 to 3) and carbachol still shifts the relationship out to higher frequencies (curve 1 to 2). For the differentiation of the two negative inotropic types under these conditions, as under other conditions, care must be taken that frequency inotropy does not accumulate against the maximal force which a preparation can generate.

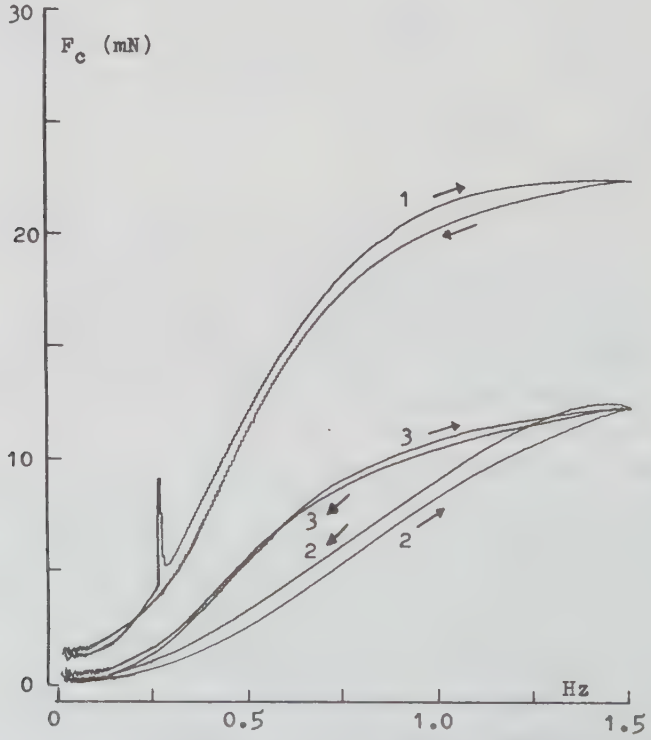


Figure 6.6. Differentiation of the negative inotropic effect of purinergic and cholinergic stimulation after ryanodine treatment plus orciprenaline. Curve 1; 4 mM Ca^{++}, 3 x 10^{-9}M ryanodine, and 10^{-5}M orciprenaline. Curve 2; as curve 1 plus 2 x 10^{-7}M carbachol. Curve 3; as curve 1 plus 6 x 10^{-5}M adenosine. 32°C. See text for further explanations.

6.7. Other conditions; effect of 8-benzylthio-cAMP.

Contractile behavior in frequency-force loops under slow response conditions at 32°C is relatively similar to behavior at 28° with ß-adrenergic stimulation. The differentiation of effects on the frequency range of frequency inotropy accumulation and the maximum of accumulation can clearly be made. In the interest of brevity, however, no curves will be presented.

The effects of cAMP-derivatives, which pass the sarcolemma, should clearly mimic the effects of ß-adrenergic stimulation according to the hypothesis outlined in the introduction of this chapter. Unfortunately, cAMP-derivatives tested to date all have "side-effects", which make their use in detailed studies problematic. Mensing (1978) reported that the positive inotropic effect of dibutyryl cAMP is accompanied with an enhancement of frequency inotropy production. This substance, however, has also negative inotropic effects in atrium, probably unrelated to stimulation of protein kinase. Whether its positive ino-

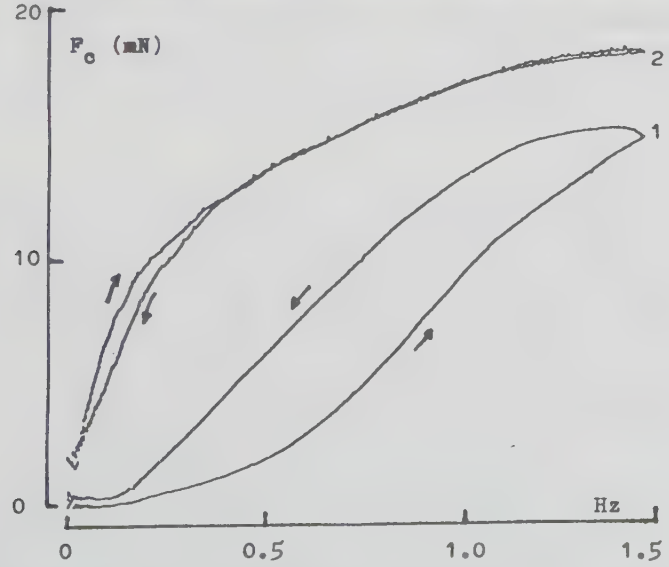


Figure 6.7. The positive inotropic effect of 2×10^{-5}M 8-benzylthio-cAMP in the presense of 3×10^{-7}M IBMX. Curve 1, 3×10^{-7}M IBMX. Curve 2, 3×10^{-7}M IBMX plus 2×10^{-5}M 8-benzylthio-cAMP. 28°C, 1.2 mM calcium. See text for further explanations.
(8-benzylthio-cAMP was a generous gift of Dr. Wachtel, Berlin.)

tropic effect is due only to stimulation of protein kinase, or to phosphodiesterase inhibition as well, is unclear.

8-benzylthio-cAMP has been shown not to raise cAMP levels in heart (Endoh and Nakamura, 1980). Therefore, if this substance mimics the effect of ß-adrenergic stimulation, certain hypothetical mechanisms of frequency inotropy can be eliminated, for example a local, beat-dependent cAMP production in the close vicinity of particulate-bound protein kinase. As the figure 6.7 shows, the effect of this substance is a marked shift of frequency inotropy accumulation to lower frequencies with very little effect on rested state contractions. The effect is clearly similar to that of ß-adrenergic stimulation, and different than that of increasing the bath calcium. Interactions with negative inotropic agents could not be studied, since the substance apparently has a toxic side-effect; after 1 and more hours application contraction force falls continually, and this effect cannot be reversed by increasing the bath calcium. Higher concentrations lead to incomplete relaxation. A hint of these effects is apparent in curve 2, in that the returning part of the frequency-force loop falls below the rising part in an intermediate force range. The experiments were performed in the presense of IBMX to block intracellular hydrolysis of the derivative, thus allowing use of smaller concentrations.

6.8. Discussion.

The differentiation of two negative inotropic types on the mechanism of frequency inotropy in atrium underscores the value of studying drug interactions. Such studies can reveal clear differences in the actions of inotropic agents which would otherwise go unnoticed. Under control conditions at 32°C the negative inotropic effects of nifedipine, adenosine and carbachol are indistinguishable (figure 4.1). Under those conditions frequency inotropy is accumulating up to 1.5 Hz in a quite linear fashion. The maximum of accumulation is not apparent, and a reduction of the maximum of accumulation and a shift of accumulation to higher frequencies give nearly identical effects on the Bowditch part of the relationship. At 28°C the saturatable nature of the mechanism of frequency inotropy

becomes visible under control conditions as a slight bowing of the Bowditch part from linear force increase with increase of frequency. Under such conditions the differentiation of negative inotropic types is just possible (no curves presented), but the differentiation only becomes marked and clear-cut in the presense of ß-adrenergic stimulation. Under these conditions the "real" maximum of frequency inotropy becomes apparent in the useable frequency range.

The different actions of adenosine and nifedipine, on the one hand, and carbachol, on the other hand, go beyond a simple description. Whereas adenosine and nifedipine act by reducing the translation of frequency inotropy into contraction force, carbachol actually reduces the amount of underlying effect produced per beat. There is good biochemical reason to ascribe the effect of carbachol on the frequency range of frequency inotropy accumulation to an inhibiting effect on the activity of the cAMP system. There exists currently wide documentation that parasympathomimetics reduce enhanced cAMP-levels in heart (for example, Keely et al.; 1978; Brown et al., 1979; and Biegon and Pappano, 1980). The most likely mechanism for this action at present is an inhibition of adenylate cyclase, direct or indirect, whereby the inhibition can be overcome by stronger ß-adrenergic stimulation (Jakobs et al., 1979). The finding, that high ß-adrenergic stimulation can overcome the effect of parasympathomimetics on adenylate cyclase, corresponds well to the functional finding that the effect of maximal ß-adrenergic stimulation on the frequency range of frequency inotropy accumulation cannot be overcome by carbachol. Under such conditions an adenosine-like effect of carbachol predominates. This second effect is probably the effect of carbachol on potassium permeability which results in action potential shortening (Johnson, 1956). Whether or not this second effect of parasymathomimetics on atrium is mediated directly or via a cGMP-dependent system (Pott, 1979) is not of crucial importance to an interpretation of the findings presented.

As pointed out in 6.7, it is unfortunate that cAMP-derivatives have side effects, which make their use limited in frequency-force studies. Perhaps a new derivative will become available in the near future, which exhibits a more stable positive inotropic effect; for the frequency-force studies of drug interactions stable effects for 2 to 3 hours are needed. Apart from this, however, functional findings could hardly fit better to the hypothesis outlined in 6.1. This is of course no proof that the proposed beat-dependent phosphorylation of slow calcium channels by the catalytic subunit of cAMP-dependent protein kinase is correct. The possibility cannot be ruled out, that a different hypothesis could as well account for the functional findings. The work presented does, however, strongly favor our hypothesis over the hypotheses that the main mechanism of frequency inotropy production in guinea pig atrium is an intracellular accumulation of calcium or sodium.

The hypothesis proposed here should as well be differentiated from the suggestion of Niedergerke and Page (1977), that a calcium-dependent protein kinase might result in a beat-dependent phosphorylation of slow calcium channels in frog atrium. The mechanism of frequency inotropy production in guinea-pig atrium is enhanced by ß-adrenergic stimulation. This effect might be accounted for in terms of a calcium-dependent phosphorylation by saying that the degree of phosphorylation per beat depends on the systolic calcium concentration. If that were the case, negative inotropic agents (nifedipine and adenosine, for example) would shift accumulation of frequency inotropy back to higher frequencies. Also, if a calcium-graded, calcium-dependent protein kinase were responsible for the production of frequency inotropy, a nearly linear accumulation of frequency inotropy with increase of frequency under control conditions at 32°C would be be hard to explain; the phosphorylation per beat would be expected to be larger, the larger the contraction.

The proposal of a depolarization-dependent phosphorylation of membrane proteins by cAMP-dependent protein kinase would not exclude the possibility that calcium in some way modulates this process. This idea is in fact supported by the finding that higher concentrations of ß-adrenergica are needed to shift frequency inotropy accumulation to low frequencies after reduction of the bath calcium than after other negative inotropic interventions. An effect of calcium on the membrane, perhaps on negative surface charges, might modulate the depolarization-dependent phosphorylation.

Depolarization-dependent phosphorylation of neuronal proteins in rat cerebral cortex has been well characterized (Forn and Greengard, 1978). The mechanism described does not, however, seem easily amenable to a depolarization-dependent phosphorylation by cAMP-protein-kinase. The described dependence of depolarization-induced phosphorylation on the presense of calcium in the medium could be explained by the above consideration. However, it is more dificult to explain why cAMP-induced phosphorylation of membrane proteins was not changed by calcium removal. It seems most probable that in that preparation depolarization-induced phosphorylation is indeed mediated by the intracellular calcium concentration. Further biochemical work, if possible in heart, is necessary to gain evidence for or against the hypothesis that the phosphorylation of membrane proteins by cAMP-dependent protein kinase depends on depolarization.

6.9. Summary of chapter 6.

1) Rested state contractions are suppressed under "slow-response" conditions in guinea pig atrium.
2) Under conditions where the rested state contractions are suppressed a "real" maxium of frequency inotropy accumulation can be differentiated.
3) ß-adrenergic stimulation shifts accumulation of frequency inotropy to a lower frequency range.
4) Carbachol can antagonize for the most part the effect of ß-adrenergic stimulation.
5) Nifedipine and purinergic stimulation reduce the maximum of frequency inotropy accumulation with no effect on the frequency range of accumulation.

7. Analysis of force transients in response to interval changes.

7.1. Introduction.

"Approaches to the analysis of the interval-strength relationship other than the ones described in this symposium (Blinks/Koch-Weser and Kruta methods) have been proposed;(..) All are attempts to place a large number of observations into some sort of ordered framework, and time will tell which approach is the most useful in doing this."

--J.R. Blinks and J. Koch-Weser (1964) at the Second International Pharmacological Meeting.

This chapter presents a detailed continuation of Blinks' and Koch-Weser's analysis of the processes underlying the production of the atrial interval-strength relationship (1961). As pointed out in the introduction to this dissertation, the last 20 years have not so much shown that one analysis method or concept is definitive or complete, but rather that interval-strength phenemena are more diverse than originally anticipated. It was of course logical and useful to attempt to fit as many observations as possible into a simple analysis system, but even in 1964 it was clear that the processes analyzed by Blinks and Koch-Weser in atrium were not identical to certain marked effects of interval change in ventricular muscle (Johnson et al., 1964). Even in atrium it was an interpretation and not an analysis to fit the effects of extrasystolic beats and post-stimulatory potentiation into the scheme of "PIEA" and "NIEA".

At present it seems safer to conclude that most important processes controlling contractility at the cellular level will be represented in some interval-strength phenomonon, rather than to conclude that nearly all interval-strength phenemena can be accounted for by two effects of activation. The contraction curve, itself, represents the quickest processes controlling contraction, and changes of the curve will represent certain changes of those processes. At the next time level there are a group of phenomena which take place quite rapidly between beats or in the course of just a few beats. The analysis methods of Kruta (1964) and Johnson et al. (1964) dealt primarily

with rapid phenomena. Finally, there are effects which take place more slowly over many beats and minutes. No single analysis method can deal with all of these phenomena, and no single analysis method can avoid a certain overlapping from one level of observation to the next.

The analysis method of Blinks and Koch-Weser provides a quantitative look at the processes responsible for the production of the steady state frequency-force relationship in atrium. The "NIEA" represents the process responsible for the production of the Kruta part of the atrial frequency-force relationship. The "PIEA" represents the process responsible for the Bowditch part of the relationship. There could be no better starting point than this for getting at the underlying mechanisms. An awareness of the complexity of the interval-strength phenomena suggests that the work presented by Koch-Weser and Blinks should not be regarded as finished or comprehensive. Also, advancements toward an understanding of the action of drugs and the development of new drugs with directed actions suggest a number of questions which might be answered by this analysis method. It is hoped that this chapter and chapter 8 may at least partially fulfill the expectations which were placed in such an analytical approach:

"The methods for quantitative analysis of the interval-strength relationship described in this paper provide a tool for the investigation of the nature of the processes responsible for the interval-dependent changes in the strength of contraction of heart muscle. They will also permit a more precise definition of the effects of drugs upon the interval-strength relationship than has previously been possible, and it can be anticipated that their use will lead to an increased understanding of the mechanisms by which drugs affect myocardial contractility."

--J.R.Blinks and J.Koch-Weser (1961)

7.2. On the limits of linearity of the contractile system.

In mammalian atrium inotropic effects of interval change and several drugs result in parallel changes of the contraction velocity and peak tension with little change of the time to peak tension (Blinks and Koch-Weser, 1964; Koch-Weser et al., 1964). Such changes reflect changes of the "degree of activation," as opposed to changes of the "duration of the active state." According to present concepts these inotropic effects must come about via increases or decreases of the amount of calcium made available for contraction activation, and peak contraction force can be taken as a quite reliable monitor of the relative amount of calcium activating contraction.

As well recognized by these authors, a contractile system will show linear changes of peak contraction for linear changes in the degree of activation only within certain limits. In the highest and lowest contractile ranges of a preparation, changes of the degree of activation will be distorted. No matter how high the degree of activation might be, a muscle can increase its force of contraction only up to a certain ceiling. These distortions probably reflect the relationship between calcium concentration and contraction force, determined by the contractile proteins. This relationship can be studied on muscle preparations in which the sarcolemma has been removed, so-called skinned muscle preparation. To obtain a picture of how this relationship might distort inotropic effects, calcium-force relationships for skinned ventricle cells presented by Fabiato and Fabiato (1975) were analyzed by fitting them to a sigmoid function:

$$F = \frac{F_{max} \cdot (Ca^{++})^n}{K^n + (Ca^{++})^n}$$

F = force
K = "binding" constant

For the curves described by these authors quite good fits are obtained with Hill coefficients (n) ranging from 2 to 4 under the various conditions described. A typical curve (pMg 4.5), plotted in linear force and calcium scales, is shown in figure 7.0.A. This curve showed an n-value of

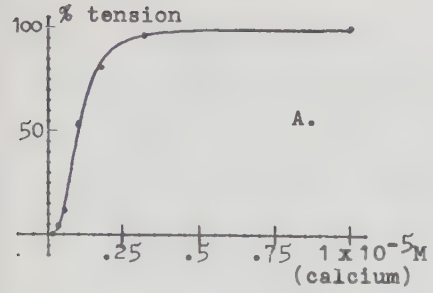
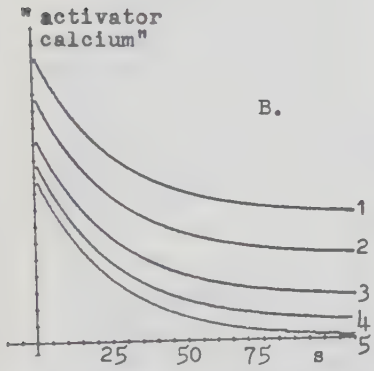
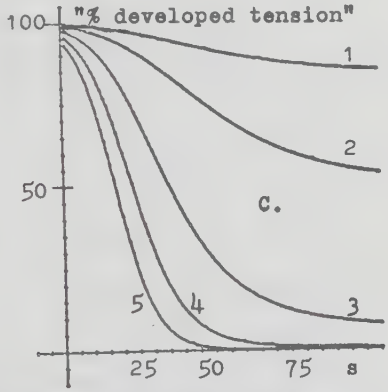


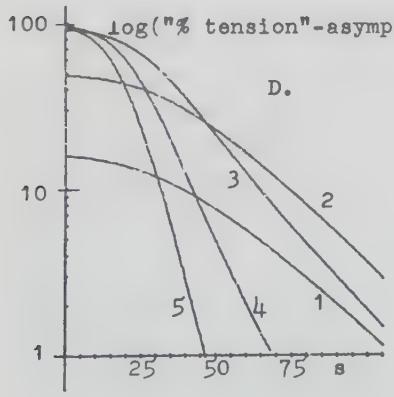
Figure 7.0. Panel A shows a typical calcium-force relationship from skinned cardiac cells (Fabiato's, 1975), fitted to a sigmoid function.

Panels B, C and D demonstrate how the calcium-force relationship would distort a linear effect on activator calcium, if the extremes of contractility are not avoided.





In panel B a hypothetical effect decays exponentially by the same amount from various high to low levels; for each curve a semi-log plot would yield the same line after subtraction of asymptotes. From B to C the calcium curves were translated into contraction force via a sigmoid function (n=4). Panel D shows the semi-log plots of the curves in C. None of the curves in D accurately reflect the underlying effect. A force transient must run between 80 and 10% of a preparation's contraction maximum for analysis without appreciable distortions.



2.9, which probably is determined by multiple factors (Potter and Gergely, 1975; Solaro and Shiner, 1976).

7.0.B, C, and D demonstrate in simulated curves how this relationship would distort a simple, exponentially-decaying effect on activator calcium under unfavorably chosen experimental conditions. In B, curves are given for a hypothetical effect on activator calcium, which decays from various high levels to various low levels with a t_{50} of 18 s. The amount of effect decaying in each of the curves 1 to 5 is identical. Thus, if these curves were analyzed by subtracting an asymptote and plotting the resulting calcium values in a semilogarithmic scale against time, exactly the same line would result for each of the curves. For the curves in C, the activator calcium transients were translated into hypothetical contraction force on a sigmoid function with an "n" of 4 and a K-value small enough so that each curve for contraction force begins at a high level. To obtain the curves in D, asymptotes were subtracted from each of the curves in C, and the resulting force values were plotted semilogarithmically against time. Drastic distortions of the ideal, single line for the original effects on activator calcium are the result.

Two things happen: 1) At the highest force levels the curves are bowed at the top, and the amount of effect decaying in curve 1 would be greatly underestimated. 2) When a force transient ends at a level less than 10% of the maximal peak tension for a preparation, the apparent rate constant determined is distorted toward an acceleration (curves 3, 4 and 5). A maximal apparent acceleration is obtained for a curve approaching zero contraction force; acceleration by a factor 4 for a Hill coefficient of 4. Note that in curves 4 and 5 the decay rate is accelerated from the true rate over the entire course of the curves, not just in the force range below 10%.

The reader should not be intimidated by these extreme distortions. If experiments are designed to keep contraction force running between 10 and 80% of the preparations' maxima, these effects can be quite completely avoided. In

the intermediate force range the rate constant determined for an effect will accurately reflect the rate constant for the effect on activator calcium; changes of the amount of effect decaying can also be reasonably accurately assessed. The figure points out clearly one of the dangers of analyzing force transients, and the literature is not free of examples of effects which may be accounted for by such distortions.

One important implication of the steepness of the force-calcium relationship has not always been appreciated. A 50% change of contraction force does not correspond to a 50% change of activator calcium amount. An increase of activator calcium by 25 to 30% in the "linear" part of the calcium-force relationship would result in a 100% increase of contraction force.

7.3. Force transients under control conditions.

7.3.1. What process determines the rate of frequency inotropy decay?

Before presenting analyzed curves it seems worthwhile to consider briefly what such curves might represent. As shown by Blinks and Koch-Weser, the process of frequency inotropy in atrium decays quite slowly (t_{50} of 40 to 90s), and the rate of decay does not depend on the rate of stimulation. According to the simplest formulation of the hypothesis that frequency inotropy reflects an accumulation of calcium in the SR (Manring, 1970), the rate of decay of the effect would be determined by the rate of calcium exchange out of the SR. In the Manring model a reduction of slow inward current in a step could exert a negative inotropic effect only as fast as the rate of decay of frequency inotropy. According to the hypothesis that frequency inotropy results from a beat-dependent phosphorylation of slow calcium channels, the rate of decay of frequency inotropy would reflect the rate of dephosphorylation of channels. If the slow inward current is directly involved in activation of contraction, a step reduction of slow inward current would result in an instantaneous negative inotropic effect.

Figure 7.1 contrasts the rate of frequency inotropy decay with the rate of the negative inotropic effect of ATP. For the experiment in the figure, frequency was increased

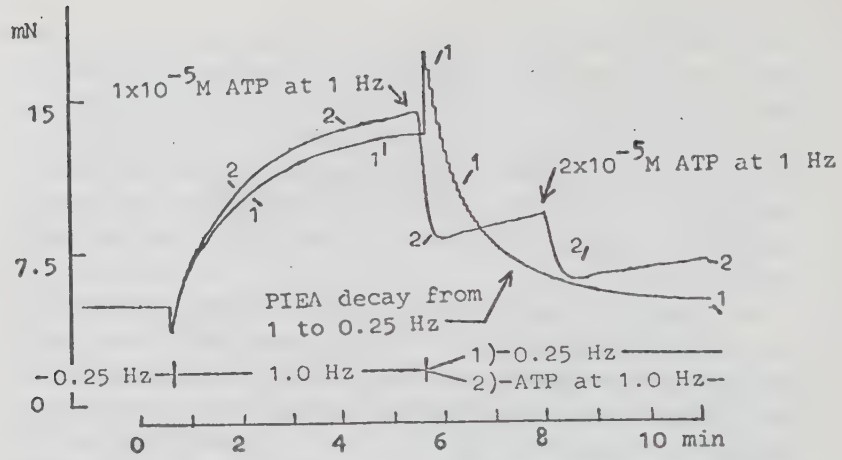


Figure 7.1. The figure contrasts the time course of frequency inotropy decay and the negative inotropic effect of ATP. 32°C; 2.5 mM calcium. The experiment is representative for over 20 similar observations. See text for further explanations.

twice from 0.25 to 1.0 Hz for 5 min. In curve 1 frequency was then decreased again to 0.25 Hz to follow frequency inotropy decay, after force overshoot in the first beat at the lower frequency. In curve 2 the stimulation frequency was left at 1.0 Hz, and 10^{-5}M ATP was added to follow the time course of the negative inotropic effect; after 2.5 min the concentration was doubled. The negative inotropic effect of ATP (or adenosine) follows a time course 5 to 10 times more rapid than the slow phase of frequency inotropy decay. The figure contradicts the simplest idea of how calcium accumulation in the SR could result in frequency inotropy production. It does not contradict the possibility that calcium accumulation in SR is the mechanism of frequency inotropy, and release of calcium from the SR for activation is graded by the size of slow inward current.

7.3.2. Frequency-dependence of force-transient form for step reduction of frequency.

Figure 7.2 presents two typical force transients for step reduction of frequency to different frequencies; the frequency jumps were made one after the other on the same preparation. The curve 1 is a"PIEA decay curve" according to

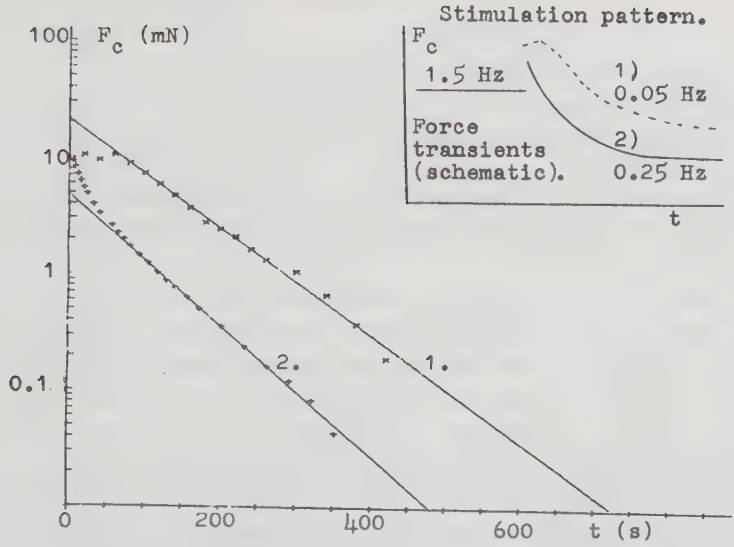


Figure 7.2. Two consecutive frequency jumps. For curve 1 frequency was reduced in a step from 1.5 to 0.05 Hz (x's). For curve 2 frequency was reduced in a step from 1.5 to 0.25 Hz (+'s). 29°C, 1.2 mM calcium. See text for further explanations.

the method proposed by Blinks and Koch-Weser: Frequency is reduced in a step from a high value (1.5 Hz) to a low value (0.05 Hz). An asymptote is subtracted for all force values in the force transient, and the resulting values are plotted semi-logarithmically against time. The first 4 force values in curve 1 were not used for curve fitting to a single exponential. The exponential curve phase shows a half-life of 70s. The strength of the first beats at the low frequency falls below the ideal level for the PIEA decay line. In terms of the Blinks/Koch-Weser analysis, this phenomenon reflects the decay of NIEA in the transient. The intercept of the PIEA decay line with the force axis at t_o is considered to be a quantitative measure of the PIEA accumulated at 1.5 Hz. The PIEA produced per beat, considered by the above authors to be constant under any given conditions for all frequency ranges, can be calculated from the analyzed curve via the following simplified formula:

$$\text{for } e^{-K/(f_1-f_2)} < 0.1; \quad \text{PIEA/beat} = \frac{PIEA_o \cdot K_{PIEA}}{(f_1 - f_2)}$$

$PIEA_o$ = curve intercept at t_o with the force axis
K_{PIEA} = rate constant of the transient ($\ln 2/t_{50}$)
f_1, f_2 = high and low frequencies chosen for frequency jump.

For the curve 1 a PIEA/beat of 0.14 mN is calculated or about 5% of the maximal force development of this preparation.

A somewhat different picture arises if the frequency jump is made to an intermediate frequency, conditions which Blinks and Koch-Weser did not analyze in detail. For curve 2 in figure 7.2 frequency was reduced in a step to 0.25 Hz, typical for over 300 such curves analyzed. The slow decay phase is preceded by more quickly decaying effects, and the size of the PIEA decay line for the slow phase is much smaller than for curve 1. With frequencies taken into account according to the above equation, the PIEA per beat in curve 2 is about 3-times less than in curve 1. It is unclear to me why Blinks and Koch-Weser did not find a dependence of the PIEA per beat on the frequency to which a jump is made, since they reported in their 1961 paper that "PIEA decay lines obtained using the same interval for cumulation of the PIEA, but various test intervals, all project back to the same point on the PIEA axis." The best way to study this dependency, I have found, is to make a frequency jump to a high frequency; then 5 or 6 beats are made at 0.25 Hz to allow the rapidly decaying effects to decay; finally, frequency is reduced to various lower levels and the decay of the slow phase is followed. Within limits, the size of the PIEA per beat of the slow phase is greater, the lower the frequency to which the jump is made (in the interest of brevity, no further curves are presented). A possible explanation for this behavior is given in chapter 8.

7.3.3. Extrasystolic beats.

One of the most dramatic effects of interval change on contractility is the effect of extrasystolic beats. In the figure 7.3 the typical effect of a single extrasystolic beat on subsequent beats in a regular beat train was stud-

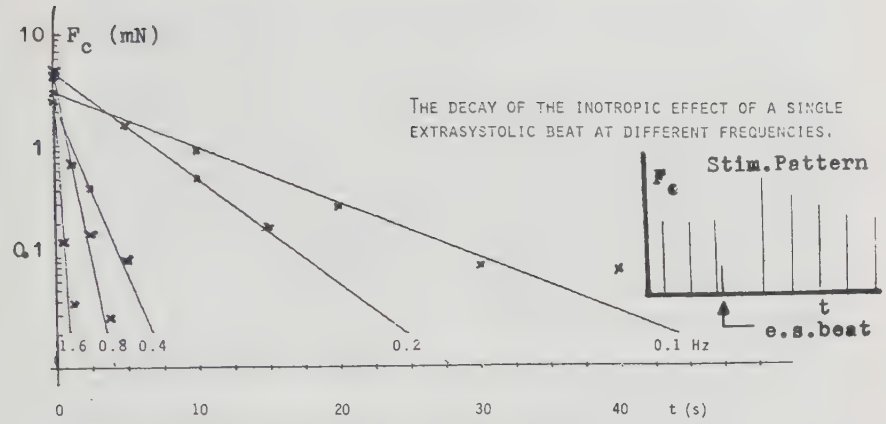


Figure 7.3. Decay of the effect of single extrasystolic beats at various frequencies. An extra beat was added about 80 ms after a regular beat, one beat at the regular interval was left out, and the fall of force back to steady state was analyzed in the subsequent beats. A steady state asymptote was subtracted, and the log of force differences was plotted against time. Note that this effect decays, within limits, in a certain number of beats, regardless of frequency. See text for further explanations.

ied. The atrium was stimulated at the given frequencies until steady contraction force was reached. Then an extra beat was added about 80 ms after a regular beat; the following beat was left out and the fall of force from the potentiated level was followed in the subsequent beats. With this stimulation pattern the potentiated state decays back to the steady state level in 4 to 6 beats, regardless of the frequency at which the effect is followed. The size of the positive inotropic effect of the extra beat is nearly the same, when a compensatory pause is not interjected after the extra beat, and stimulation is continued immediately, so that the interval of the first beat after the extra beat is 80 ms less than the regular interval. However, under such conditions the resulting force transient also picks up some of a quickly-decaying, beat-independent effect, making its analysis more complicated than in the figure (no curves presented, the quick beat-independent will be described).

Two observations seem most important: The positive inotropic effect of extrasystolic beats is very large, 7 to 15 times larger than the "PIEA per beat" of Blinks and Koch-Weser; the slow PIEA produced per beat is so small that it has practically no bearing at all on the effect of single extrasystolic beats. The effect of extrasystolic beats is more intimately related to the process of activation than is the slow PIEA; whereas the slow PIEA decays between beats, the effect of extrasystolic beats is dissipated by the beat itself,as is evident in its beat-dependent decay kinetics.

7.3.4. A rapid, beat-independent phase of frequency inotropy decay.

The question arises, whether the effect decaying after extrasystolic beats represents the same effect seen in the onset of force transients for frequency reduction from a high to intermediate frequencies (curve 2 of figure 7.2). If this were the case, the rapid decay phase in frequency jumps should show a beat-dependent kinetics. That is, the decay rate of this effect should depend on the frequency to which the frequency jump is made. Figure 7.4 demonstrates that this is <u>not</u> the case, typical for 5 similar experiments. On the contrary, there clearly exists a quickly-decaying effect in force transients for frequency reduction in the intermediate range, which is beat-<u>independent</u>. In figure 7.4 the rapidly-decaying phase of frequency inotropy shows roughly the same rate constant, whether the jump is made from 1 to 0.25 Hz or to 0.5 Hz. There are a number of ways to demonstrate the existence of this effect, independent of the beat-<u>dependent</u> effect of extrasystoles; one method is presented in figure 7.9. The possibility remains, and will be varified later, that the quick phase of frequency inotropy decay seen in jumps as in figure 4 results from the combination of a beat-dependent effect, related to the effect of extrasystolic beats, and a beat-independent effect which decays at about the same rate under such conditions.

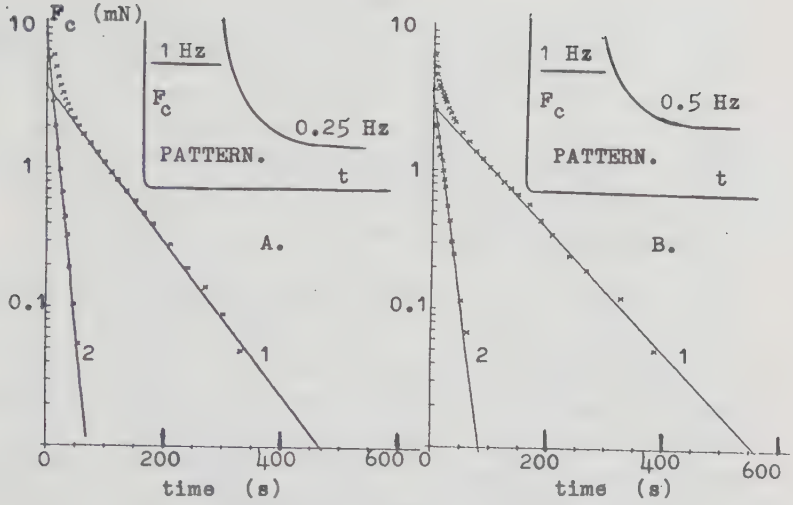


Figure 7.4. The figure demonstrates the presense of a rapid phase of frequency inotropy decay, which decays beat-independently with a half-life of 7 to 12 s. In panel A frequency was reduced in a step from 1.0 to 0.25 Hz, in panel B from 1.0 to 0.5 Hz. Force transients were fitted to two exponential functions by the least-squares method:

$$F_c = Xe^{-k_1 t} + Ye^{-k_2 t} + Z.$$

The quick phase (2) is obtained by subtracting the asymptote and the slow phase (1) from the force transient. Under the conditions of this experiment the quick and slow phases are separated kinetically by a factor 5 to 10 (28°C; 1.2 mM calcium). This degree of separation allow fairly accurate curve-fitting.

Parameters for the given curves are:

	X	k_1	Y	k_2	Z
A.	3.8	0.013	9.0	0.095	1.5
B.	2.9	0.014	2.2	0.066	2.9

For the given experiment the quick phase of frequency inotropy decay is in fact somewhat more rapid in the curve decaying at 0.25 Hz than at 0.5 Hz ($k=\ln 2/t_{50}$).

There clearly exists a rapidly-decaying phase in force transients, which decays beat-independently. It can also be differentiated in appropriately designed force transients for frequency increase (no curves in the interest of brevity).

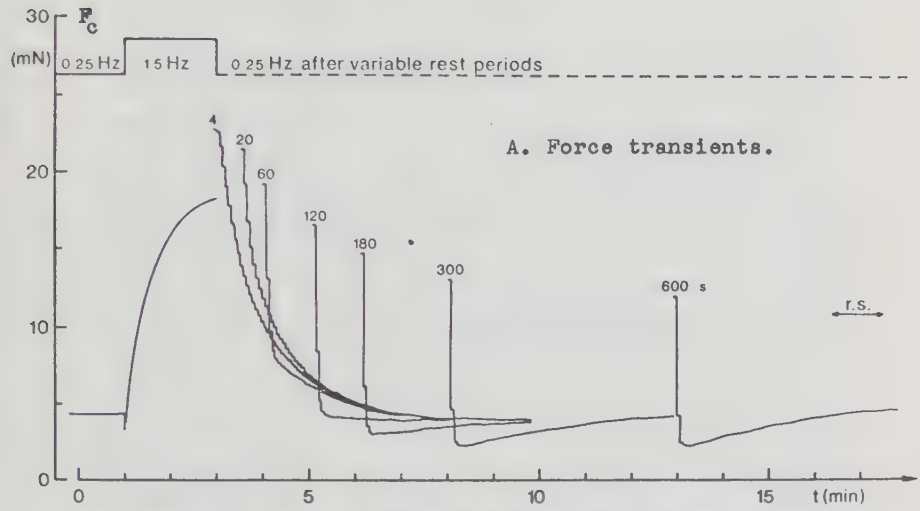
In order to talk about the described effects, I have adopted the terminology, "PIEA's I, II, and III." PIEA I refers to the most apparent slow phase of frequency inotropy, the process analyzed by Blinks and Koch-Weser. PIEA II refers to the rapid, beat-independent phase of frequency inotropy. PIEA III refers to the beat-dependent, post-extrasystolic effects and corresponding effects which might be differentiated in frequency jumps.

7.3.5. Frequency jumps series: The relationship of PIEA III to PIEA I and to the "NIEA." Differentiation of PIEA 0.

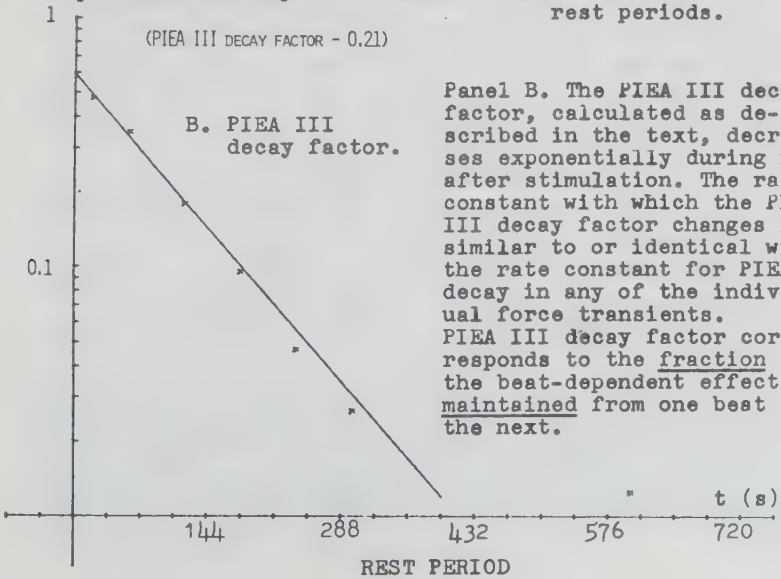
A good deal more information can be gathered by analyzing not one frequency jump, but a series of jumps. Figure 7.5 presents one of the series-types which reveals interesting relationships between the underlying processes. For this type of jump series, frequency is increased from a low level (0.25 Hz) to a high level (1.5 Hz) for long enough to allow accumulation of a good deal of frequency inotropy (2 min), but short enough to avoid the effects of metabolic fatigue. After stimulation at the high frequency for the 2 min, stimulation is ceased for various time periods, and then restarted at the low frequency (0.25 Hz). The procedure is repeated in randomized order with 5 to 10 different rest periods. During this kind of jump series the preparations show a remarkable stability; the 7^{th} to 10^{th} force transients for increase of frequency to 1.5 Hz are nearly identical to the 1^{st} one. During rest periods of more than 15 s most of the PIEA II dissipates, so that the resulting force transients contain for the most part only a rapid, beat-dependent phase (PIEA III) and the PIEA I.

The PIEA I behaves quite exactly as would be expected for a simple, linear process. In the single force transients for 4, 20 and 60 s rest, the PIEA I decays with a half-life of about 60 s. In such a force transient the PIEA I is not decaying toward zero, but toward the given steady state level at 0.25 Hz; during rest however it is decaying toward zero or its rest level. After 120 s rest

Figure 7.5. Frequency jump series. 32°C; 2.5 mM Ca^{++}



Panel A. Force transients from a typical frequency jump series for the given stimulation pattern. Frequency is increased from 0.25 to 1.5 Hz for 2 min; then a rest period is interjected, and stimulation is resumed at 0.25 Hz. The procedure is repeated several times with different rest periods.



Panel B. The PIEA III decay factor, calculated as described in the text, decreases exponentially during rest after stimulation. The rate constant with which the PIEA III decay factor changes is similar to or identical with the rate constant for PIEA I decay in any of the individual force transients.
PIEA III decay factor corresponds to the fraction of the beat-dependent effect maintained from one beat to the next.

the PIEA I acummulated at 1.5 Hz has decayed to 75% of its final rest level (t_{50}= 60 s). This corresponds quite exactly to its steady state level at 0.25 Hz, so that the force transient at 120 s rest arrives at its level of equilibrium after the 5 or 6 beats needed for the beat-dependent PIEA III to reach its steady state. During still longer rest periods the PIEA I decays to a level below the steady state level at 0.25 Hz, and therefore it reaccummulates after resumption of stimulation. The amount of PIEA I present in the preparation at the first beat after rest can be determined by analyzing the individual force transients; it clearly decreases during rest with the same rate constant as the PIEA I decays during individual frequency jumps (no curves presented).

The feature of this frequency jump series which is of most interest concerns the quick phase of force decline seen in each of the force transients after resumption of stimulation at 0.25 Hz. This phase of the transients can be shown to be strongly beat-dependent, like the decay of the effect of extrasystolic beats. That is, for any of the given conditions (= period of rest after stimulation) the quick phase needs a certain number of beats, not a certain amount of time, to decay. For force transients in which little PIEA I decays, a decay factor for the quick phase can be determined by simply measuring the force differences from one beat to the next. The fraction of the effect which is maintained from one beat to the next (= PIEA III decay factor) is given by:

$$\frac{dF_2}{dF_1} = \frac{dF_3}{dF_2} = \frac{dF_4}{dF_3} \ldots = \text{PIEA III decay factor}$$

The fraction of the effect lost from one beat to the next is 1-PIEA III decay factor.

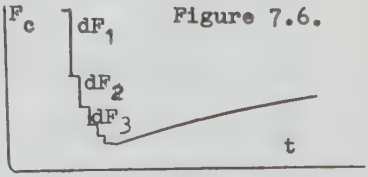


Figure 7.6.

For frequency jumps in which the PIEA I is still quite large, the entire force transient must be analyzed by the least squares method, and the PIEA III decay factor can be determined from the quick phase after subtraction of the PIEA I.

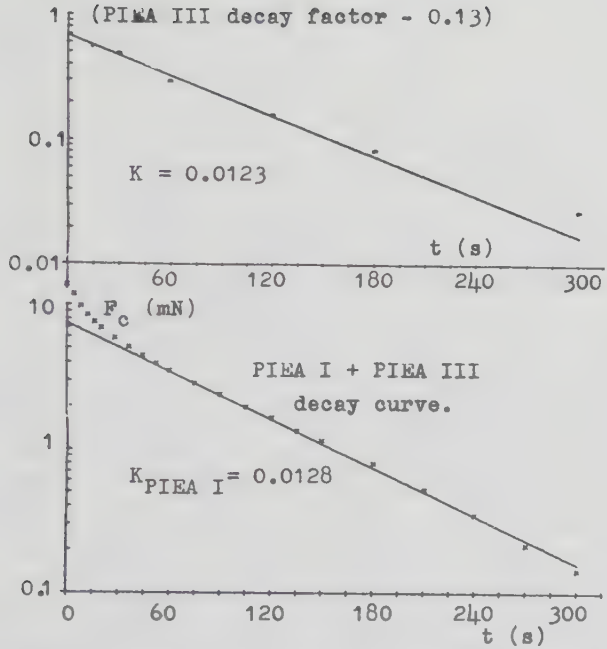


Figure 7.7. Upper panel: Decay of PIEA III decay factor as analyzed from a frequency jump series. Lower panel: A single force transient after a 20 s rest period, analyzed from the same jump series. 28°C; 1.2 mM Ca^{++}. Representative for 6 similar experiments. See text for further explanations.

In a frequency jump series the PIEA III decay factor decreases exponentially with period of rest after stimulation. That is, after longer rest periods less beats are needed for the PIEA III to come to equilibrium. As shown in panel B of figure 7.5 the PIEA III decay factor decreases from about 0.7 immediately after stimulation at 1.5 Hz to 0.2 at the rested state under the given experimental conditions. The rate of change of the PIEA III decay factor during rest is very similar to the decay rate of the PIEA I taken from an individual force transient. For 7 experiments taken as a whole, there was no significant difference between the decay rates of the PIEA I and the PIEA III decay factor, although the former rate "tended"to be slightly greater than the latter.

In figure 7.7 the decay of the PIEA III decay factor from a frequency jump series at 28°C is compared to the decay of frequency inotropy from a single frequency jump. The PIEA III decay factor at the rested state (= assymptote of the decay curve) is quite strongly temperature-dependent. A factor of 0.13 is representative at 28°C; it increases to about 0.3 at 37°C. The finding, that the PIEA III decay factor changes with the amount of accumulated frequency inotropy, is one of the findings which was predicted by the electro-mechanical coupling model presented in chapter 8 before the effect was actually measured.

The term, PIEA III, is not completely satisfactory. On the one hand, this effect contributes to the production of frequency inotropy, and it decays with frequency reduction from a high frequency to an intermediate frequency. The decay of the positive inotropic effect of extrasystolic beats corresponds to the same process. From this perspective it is a positive inotropic effect of activation. On the other hand, this is the same process responsible for loss of contractility with increase of frequency from the rested state (= "NIEA"). The term, PIEA III, makes sense from all perspectives, only if one considers the rested state of atrium as a condition in which a certain amount of "positive effect"has accumulated. On stimulation this effect decays or is lost, and it then can be regained at high frequencies.

There are other ways of examining the dependence of the PIEA III decay factor on the amount of frequency inotropy. One way is to increase the frequency for various time periods, and then examine force transients at a low frequency (0.25 Hz) after a constant 15 to 20 s rest period to allow the PIEA II to decay. The PIEA III decay factor is clearly greater, the larger the amount of frequency inotropy which is allowed to accumulate (no curves presented). It seems safe to conclude that the PIEA III decay factor depends directly on the amount of PIEA I present in a preparation; the PIEA III decays more slowly in the presense of more PIEA I.

The PIEA III is strictly beat-dependent only within

limits in atrium. Under controlled conditions the PIEA III decays in less beats at higher frequencies. For example, contraction force falls in 4 to 6 beats after a rested state contraction with stimulation at 0.25 Hz. Only 2 or 3 beats are needed for the same PIEA III decay with stimulation at 1.0 Hz. The decay of PIEA III is overly beat-dependent. This statement applies to the effect of extrasystolic beats at various frequencies, to the fall of force after start of stimulation from the rested state, and to the PIEA III decay factor measured immediately after high-frequency stimulation (no curves presented).

A frequency jump series as in figure 7.5 allows the determination of one other curve of interest, the so-called rest-decay curve. This curve simply describes over time the force of contraction of the first beat following stimulation. At the longest rest periods the curve approaches the rested state contraction level. For atrium the curve has only seldomly described in the literature. Figure 7.8 shows a typical rest-decay curve from a frequency jump series as in figure 7.5 (32°C; 2.5 mM calcium), representative for 7 similar ones. The strength of the rested state contraction was subtracted from all force values, and the resulting force values are plotted semi-logarithmically against time.§ For the first 5 to 20 s after stimulation the strength of the first beat is greater than at 1.5 Hz. Then contraction force begins to fall toward the rested state level. It is noteworthy in all rest decay curves which I have determined for atrium, that the declining part of the curve does not simply reflect the decay of PIEA I. The PIEA I decays with a half-life of approximately 60 s, so that after 360 s it has decayed by about 99%. The rest decay curve, however, continues to fall more slowly toward the rested state with a half-life of 180 to 300 s. Obviously, some process other than the PIEA I is reflected in this curve. Such a slowly decaying effect is found in force transients only under very

§The logarithmic time scale used often to plot this curve in the literature is unwieldy; it makes it impossible to evaluate the real curve form. In my opinion its use should be discontinued.

special pharmacological conditions (to be described). To be able to talk about this effect, it is called PIEA 0. The PIEA 0 can be suppressed from the rest-decay curve by increasing the bath potassium from 6 to 9 mM. This and other findings to be presented give some reason to think that the PIEA 0 could reflect a slow loss of sodium during rest.

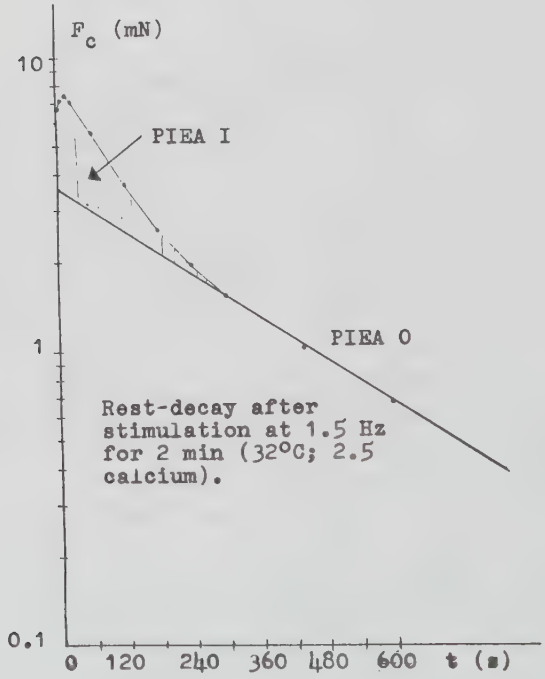


Figure 7.8. Rest-decay curve after stimulation at 1.5 Hz for 2 min as in the figure 7.5. 32°C; 2.5 mM Ca^{++}. Rest-decay in guinea pig atrium shows the presense of an effect (PIEA 0), which decays 3 to 5 times slower than the PIEA I. This effect is not normally present in force transients for frequency reduction; the PIEA 0 is for some reason only found in the rest-decay curve. For the curve presented, the rested state force was subtracted from all force values and the log of the resulting values is plotted against the rest period. Note: Atrial rest-decay can be established accurately only when the rested state value is considerably less than the stimulated level (<35°C; < 3 mM calcium).

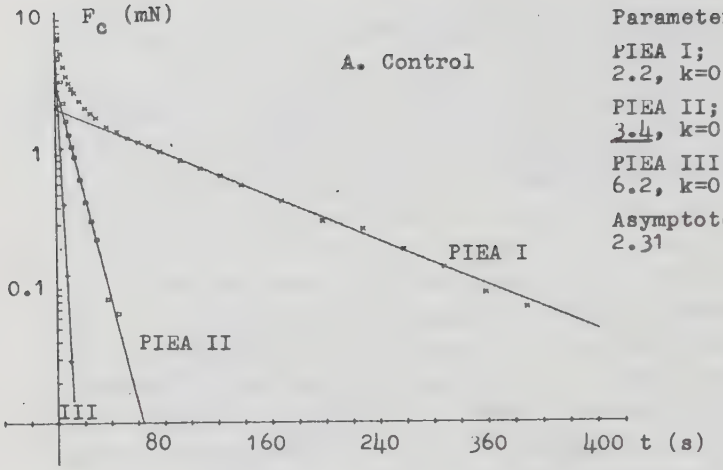
7.4. Pharmacological influences on force transients.

7.4.1. Specific inhibition of the PIEA II by 2,4-dinitrophenol.

With regards to a continued analysis of force transients, the findings on the behavior of the PIEA III opened possibilities for getting a closer look at the PIEA II. It may be projected from the findings on PIEA III, that this effect will be decaying with an apparent half-life of about 6 s in a simple frequency jump from 1.0 to 0.25 Hz. This is nearly the same time course expected for the beat-independent PIEA II, as demonstrated in figure 7.4. By exploiting the beat-dependence of PIEA III and its dependence on the PIEA I, it is possible to devise frequency jumps in which the PIEA's II and III are well separated.

One set of conditions which allows this separation is the following: A frequency jump is made from 0.5 to 1.5 Hz for just 1 min. Then frequency is reduced again to 0.5 Hz, and the resulting force transient is analyzed. For two reasons the PIEA III adjusts to the lower frequency very rapidly in this jump. First, the amount of frequency inotropy accumulated in the 1 min frequency jump is only intermediate, so that PIEA III decay will be relatively rapid. Second, since the PIEA III depends on the number of beats made, it will decay twice as fast at 0.5 Hz as at 0.25 Hz. Figure 7.9.A shows the analysis of typical force transients under these conditions. Note that the PIEA's I, II and III can be quite cleanly separated in a single transient; PIEA II is about 9 times faster than PIEA I, and the PIEA III is about 5 times faster than the PIEA II.

In the course of this work I looked at a large number of inotropic interventions in the hope of specifically inhibiting or enhancing the individual frequency inotropy phases. Generally, the PIEA's seem to change in parallel; when one is reduced or enhanced they all change in a similar fashion. One clear exception to this general pattern is the effect of the mitochondrial uncoupler, dinitrophenol. In 6 experiments the PIEA II was reduced



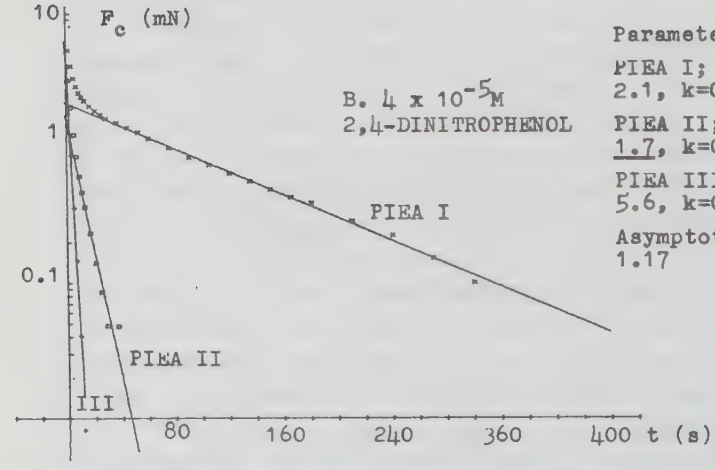


Figure 7.9. Effect of 2,4-dinitrophenol on force transients with separation of PIEA's I, II and III. Frequency jump was from 0.5 to 1.5 Hz for 1 min, and back to 0.5 Hz. The force transient for frequency reduction was analyzed by fitting it to three exponential functions with the least squares method. The PIEA II line is obtained by subtracting the PIEA I. The PIEA III line is obtained by subtracting the PIEA's I and II. 28°C; 1.2 mM calcium. See text for further explanations.

The parameters given refer to the intercepts of the exponentials at t_o in mN and the decay rate constants in s^{-1} (= ln 2/ t_{50}).

to a much greater extent than the other phases by dinitrophenol in concentrations of 2 to 6 x 10^{-5}M. In the experiment given in figure 7.9 the effect of dinitrophenol can be taken from the table of curve parameters obtained by fitting the curves to three exponentials; the PIEA II is reduced by about 50% by dinitrophenol in a concentration which has little effect on the other PIEA's. Higher concentrations have effects as would be expected for a metabolic poison; contractility is maintained at the lowest frequencies, but when frequency is increased in a step to a high level force rises shortly and then falls. Neither cyanide or anoxia had comparable specific effects on the PIEA II. For that matter, no other intervention from a list of about 20 had similar effects on the PIEA II. This supports the idea that the effect of dinitrophenol on PIEA II is quite specific.

It is tempting to interpret results as an indication that the PIEA II corresponds to handeling of calcium by the mitochondria. An uncoupler of oxydative phosphorylation would be expected to have more specific effects than other mitochondrial poisons, since an uncoupling action would destroy the proton gradient necessary for uptake of calcium (Bygrave, 1978). The possibility that some other action of dinitrophenol is responsible for its effect on PIEA II cannot be ruled out.

7.4.2. Effects on the PIEA I.

The work presented in this section was done before certain of the perspectives presented in the previous sections were gained. As a result, force transients were studied under conditions which do not allow separation of the PIEA's II and III. The quick phase of frequency inotropy decay seen in the following analyzed curves probably represents a mixture of the two effects. Therefore, only information about the PIEA I can be accurately taken from this work. Generally, simple frequency jumps from 1 to 0.25 Hz were made and analyzed. At some future date it would be important to repeat this work with an addition of a 20 s stimulation pause before reducing the frequency to 0.25 Hz. This would allow the clean analysis of the PIEA's I and III with no interference from the PIEA II.

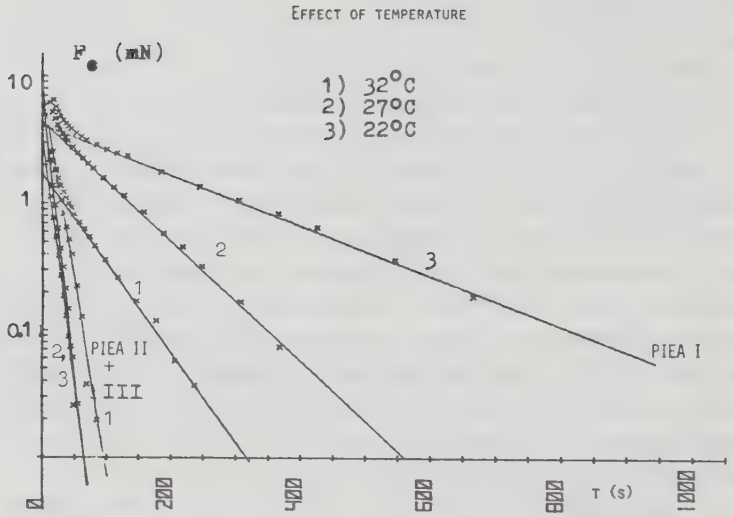


Figure 7.10. The representative effect of bath temperature on force transients for frequency reduction from 1.0 to 0.25 Hz. 1.2 mM Ca^{++}. The PIEA I phase is markedly slowed by temperature reduction. The quicker phase of force transients shows little change.

7.4.2.1. Effect of the bath temperature.

Force transients for frequency jumps from 1.0 to 0.25 Hz at 1.2 mM calcium were made at three differenct temperatures (32°C, 27°C and 22°C) in randomized order on 6 preparations and analyzed by fitting them to two exponentials. Representative curves from a single preparation are given in figure 7.10. The quick phase (PIEA's II + III) is obtained by subtracting an asymptote and the PIEA I from the force transient. The PIEA I is markedly slowed by temperature reduction and shows a Q_{10} of 2.7 ± 0.5 (SD, n=6) for the temperature range from 32°C to 22°C. Such a temperature dependence would be expected for a process which is enzymatic in nature. A practical implication of the findings is that the quick and slow phases of frequency inotropy decay are best separated at lower bath temperatures. Therefore, most experiments were performed at 28°C.

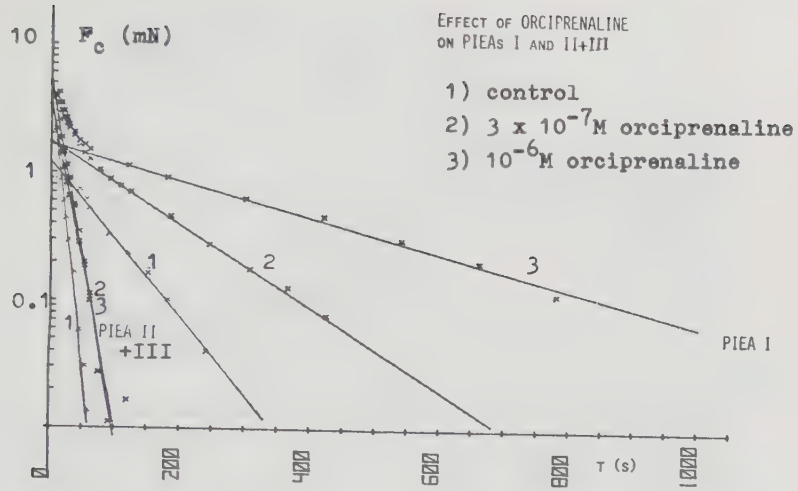


Figure 7.11. Effect of ß-adrenergic stimulation on the quick and slow phases of frequency inotropy decay. PIEA I is markedly slowed by small and intermediate concentrations of orciprenaline. Jumps are from 1.0 to 0.25 Hz. 28°C, 1.2 mM calcium.

7.4.2.2. Effect of orciprenaline on PIEA I.

As is apparent in the curves presented by Koch-Weser et al. (1964), a good part of the stimulatory effect of adrenergic stimulation on PIEA may be due to a slowing of the decay rate (about 40% due to slowing, and 60% to an increased PIEA/beat). Never-the-less, these authors concluded from their work that the main effect was due to an increase of the PIEA per beat. I have examined the effect of ß-adrenergic stimulation under several conditions, and in each case there was a marked slowing of the PIEA I. For the conditions of figure 7.11 the PIEA I was slowed from a mean t_{50} of 57 s for the control curves to 91 s with 3 x 10-7M orciprenaline ($p<0.05$; n=6), and from 91 to 175 s by increasing the orciprenaline concentration to 10^{-6}M ($p<0.01$; n=6). Also, the decay rate of the quick phase is significantly slowed at the higher concentration ($p<0.02$).

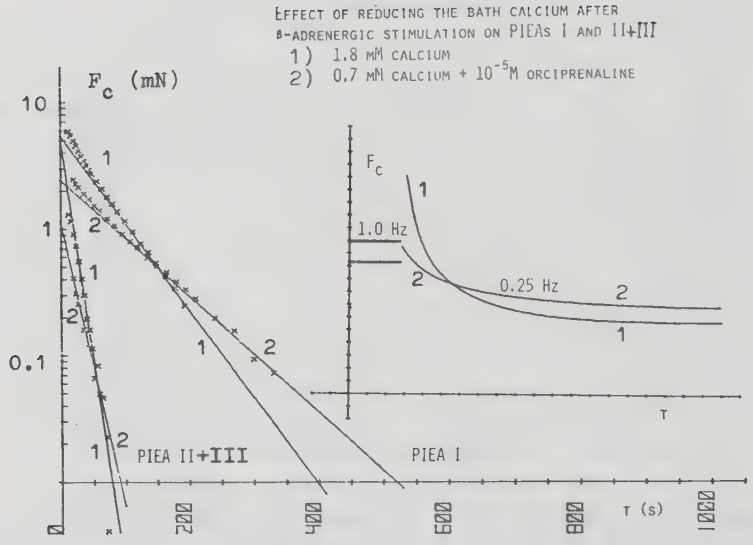


Figure 7.12. Effect of antagonizing the positive inotropic effect of orciprenaline by reducing the bath calcium. Curve 1 is a control. Curve 2 was made after addition of 10^{-5}M orciprenaline and reduction of the bath calcium from 1.8 to 0.7 mM. The slowing effect of ß-adrenergic stimulation on both the slow and rapid phases of frequency inotropy decay is maintained after the calcium reduction, although the size of the PIEA's in the frequency jumps from 1.0 to 0.25 Hz is considerably reduced under the conditions of curve 2.

Although the slowing of PIEA I by ß-adrenergic stimulation is significant, the effect might theoretically be due to some unclear effect of increasing contractility per se. This possibility is discounted by the finding that changes of the bath calcium do not effect the decay rate (no curves). As demonstrated in figure 7.12, the slowing effect of ß-adrenergic stimulation on PIEA I is maintained after antagonizing its positive inotropic by reducing the bath calcium. Note that the size of force transients in curve 2 reflects a flattening of the frequency - force relationship in the frequency range between 0.25 and 1.0 Hz, as presented in chapter 4 for other interactions with orciprenaline. Since the curve 2 is slowed in just the same contractile range in which the curve 1 runs, it may be concluded that the slowing effect of ß-adrenergic stimulation on PIEA is <u>real</u>.

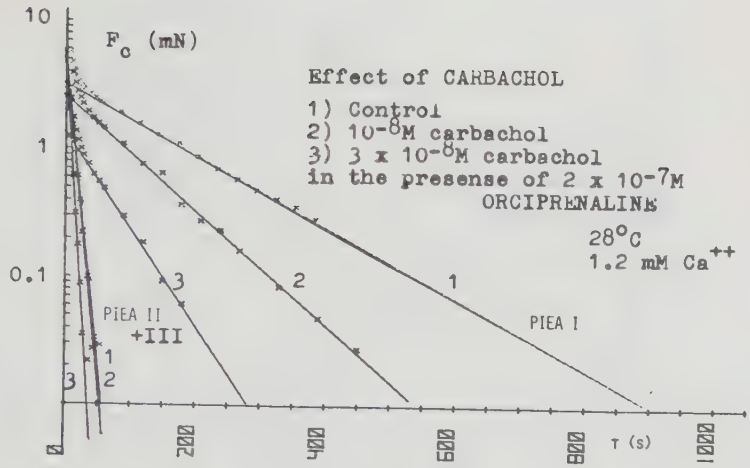


Figure 7.13 Effect of carbachol on the quick and slow phases of frequency inotropy decay in the presense of B-adrenergic stimulation. When the PIEA I has been slowed by orciprenaline, it is reaccelerated by carbachol. Frequency jumps are from 1.0 to 0.25 Hz.

7.4.2.3. Effect of carbachol on PIEA I.

In the curves presented by Koch-Weser et al.(1964) the reduction of PIEA by carbachol is accounted for to 50% by an acceleration of the PIEA decay and to 50% by a reduction of the PIEA per beat. I also have found acceleration of the PIEA I decay rate by carbachol in guinea pig atrium. It is however dangerous to study negative inotropic effects on frequency jumps from 1 to 0.25 Hz, since the steady state contraction force at 0.25 Hz may well decrease to a level less than 10%of the preparations' maxima and in this way automatically lead to a pseudo-acceleration of the decay rate. Under some conditions where this distortion was avoided, for example with increased bath calcium, little or no acceleration was found. However, if the PIEA I is first slowed by orciprenaline, as in figure 7.13, carbachol always reaccelerates the decay rate (5 similar experiments). Thus, the effect of carbachol as well as orciprenaline is mixed; both the PIEA per beat and the decay rate can be affected.

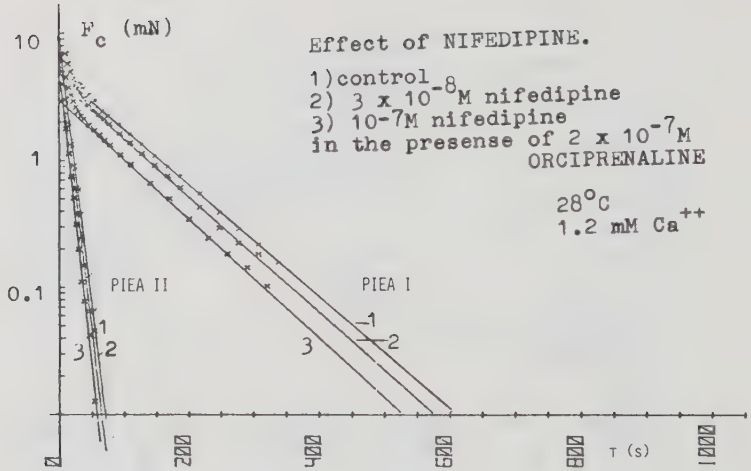


Figure 7.14. In contrast to carbachol, the "calcium antagonist," nifedipine, reduces the PIEA I without acceleration. Frequency jumps are from 1.0 to 0.25 Hz.

7.4.2.4. Effect of nifedipine on PIEA I.

Most other negative inotropic substances do not accelerate the PIEA I deday rate. This suggests that the effect of carbachol is real and quite specific. Figure 7.14 presents the typical effect of nifedipine, which was examined in 7 similar experiments. Nifedipine never changes the rate of PIEA I decay, but reduces the PIEA I size.

In conclusion, I would like to correct Koch-Weser, Berlin and Blinks' statements about the actions of carbachol and adrenergic stimulation on the atrial PIEA. Considerable portions of these agents' effects on the PIEA (I) are due to an action on the rate of decay. An exact assessment of the degree to which their effects are due to a change of rate or a change of the PIEA per beat is difficult due to non-linearities of the contractile system and probably of the PIEA mechanism itself, as well§.

§ In simulated curves, whereby the PIEA I is assumed to correspond to a beat-dependent phosphorylation of slow calcium channels, the rate of PIEA I in appropriate frequency jumps becomes"accelerated" when the phosphorylation per beat is quite large. This is due to the saturatable nature of the system, and such an apparent acceleration with strong B-adrenergic simulation is also found in real frequency jumps.

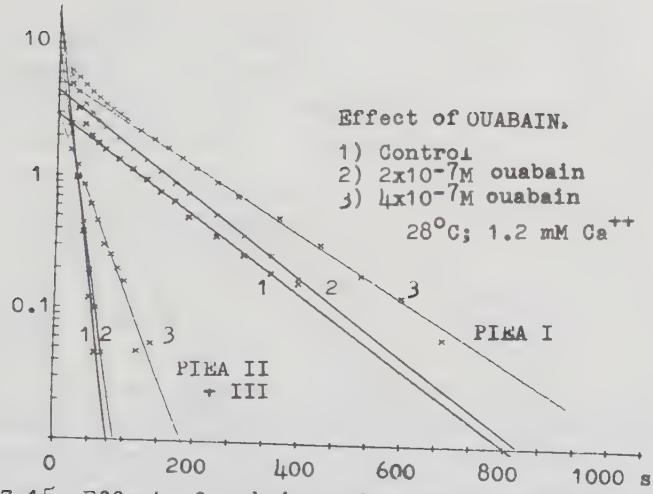


Figure 7.15. Effect of ouabain on frequency jumps from 1.0 to 0.25 Hz. Under the conditions of these experiments the size of the PIEA I is increased. PIEA I decay is not slowed. The quick phase of frequency inotropy decay is slowed by higher concentrations of ouabain.

7.4.2.5. Effects of heart glycosides.

The effect of heart glycosides on force transients is important with reference to the discussion that sodium might accumulate in heart preparations with increase of frequency and produce frequency inotropy. Since heart glycosides are generally thought to act by inhibiting active sodium extrusion, it would be expected from the above hypothesis that PIEA decay be slowed by these substances. As originally shown by Koch-Weser et al. (1964), and as verified in my own work, ouabain does not slow the normal slow phase of frequency inotropy decay (see figure 7.15) in atrium. In contrast to the findings of Koch-Weser et al., under the conditions of my experiments there is an increase of the size of the PIEA I intercept. This is remarkable, since the action of ouabain on the frequency-force relationship in atrium is for-the-most-part frequency independent; the positive inotropic effect may be greater at lower frequencies than high frequencies. A possible explanation for this behavior will be suggested in chapter 8. Intermediate to high concentrations of ouabain always slow the quick phase of frequency inotropy decay. This last effect I have studied in detail; it is due to a slowing of PIEA III decay.

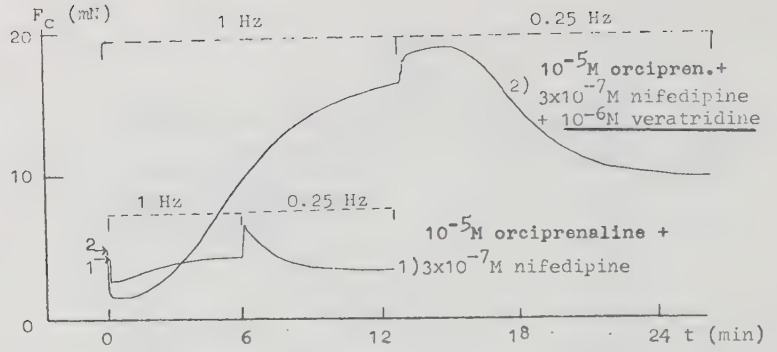


Figure 7.16. Effect of veratridine after near abolition of the PIEA I. The atrium was pretreated with 10-5M orciprenaline and 3 x 10-7M nifedipine to largely abolish the normal mechanism of frequency inotropy (see frequency jump 1 from rested state to 1 Hz for 6 min and then to 0.25 Hz).After veratridine treatment for 1h (10^{-6}M) a new mechanism of frequency inotropy is produced which accumulates and decays 3 to 4 time slower than the PIEA I (see curve 2; frequency jump from the rested state to 1 Hz for 12 min and then to 0.25 Hz).

7.4.3. Effect of veratridine.

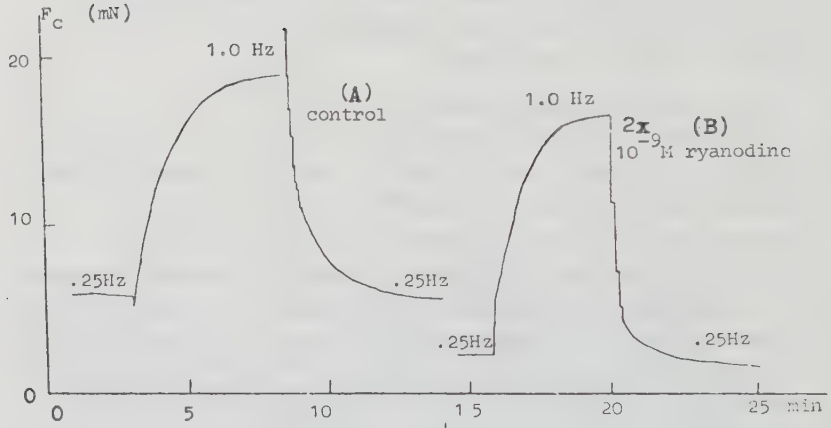
Veratridine is thought to exert its positive inotropic effect in heart by slowing the deactivation of fast sodium channels and thereby effecting an increase of intracellular sodium (Honerjäger and Reiter, 1975). If a phase of frequency inotropy production were due to an intracellular accumulation of sodium, veratridine should potentiate this phase. The action of veratridine in atrium is to create a new phase of frequency inotropy accumulation and decay. This is demonstrated in figure 7.16 under conditions where the normal PIEA I is nearly abolished by ß-adrenergic stimulation and nifedipine. Also under control conditions veratridine treatment results in a phase of frequency inotropy accumulation and decay much slower than the PIEA I. It would be logical to conclude that the kinetics of this effect corresponds to the rate of sodium extrusion in atrium at rest. The time course of this effect in frequency jumps is roughly similar to the time course of the PIEA 0 (figure 7.8). The findings do <u>not</u> support the idea that one of the normal phases of frequency inotropy decay analyzed

in frequency jumps reflects sodium extrusion. The idea is favored, that the intracellular sodium concentration in atrium is regulated so that it does not markedly increase with increase of frequency; veratridine might greatly enhance a normally-existing tendency for sodium to increase with increase of frequency.

7.4.4. Effects of ryanodine.

A number of observations led me to think that ryanodine might specifically inhibit one of the fast phases of frequency inotropy decay. The quick PIEA's always seem to be associated with the phenomenon of post-stimulatory potentiation (PSP: one-beat rise of force with increase of stimulation interval). Since ryanodine inhibits PSP (Furchgott, 1964), it seemed likely that the fast phases of frequency inotropy would as well be inhibited. This is apparently not the case. Ryanodine, like veratridine, seems to add something essentially new to frequency jumps, and this action cannot be clearly accounted for in "PIEA" terms.

The typical effect is presented in figure 7.17: Under control conditions frequency jumps from 0.25 to 1 Hz and back to 0.25 Hz are relatively symetrical. After ryanodine treatment (2×10^{-7}M for 15 min, followed by washout and continuous application of 2×10^{-9}M) frequency jumps become much more markedly asymmetrical. For increase of frequency from 0.25 to 1.0 Hz force rises quite quickly in a few beats, but the force transient is dominated by the accumulation of the normal PIEA I. In the force transient back to 0.25 Hz, however, force falls very drastically in the first few beats, and the size of the PIEA I in the transient for reduction of frequency is greatly reduced in comparison to control conditions. In strong contrast to control conditions, the force of the first beat after cessation of stimulation is greatly reduced in just a few seconds (described by Furchgott, 1964). Also in strong contrast to control conditions, the rested state contractions cannot be greatly enhanced by doubling the bath calcium or strong ß-adrenergic stimulation. (no curves presented). As usual, strong ß-adrenergic stimulation precludes the accumulation of PIEA I in frequency jumps;



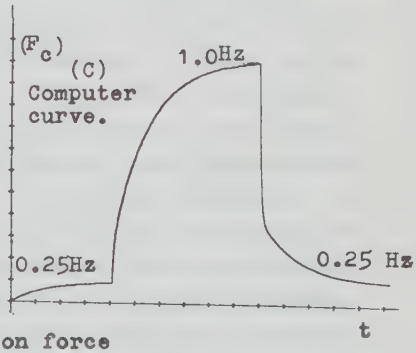


Figure 7.17.

The upper panel of the figure presents the typical effect of ryanodine treatment (2x10-7M for 15 min, followed by wash-out and continuous application of 2x10-9M) on frequency jumps. 1.2 mM Ca^{++}; 28°C. With ryanodine (curve B) the frequency jumps become markedly asymmetrical. For increase of frequency contraction force rises in a rapid, but small phase, followed by accumulation of the PIEA I. For frequency reduction force falls rapidly and the PIEA I appears to be reduced greatly.

The curve in the lower panel demonstrates how such an asymmetry might come about. A rapid and a slow, linear effect were simulated. A certain amount of each effect is produced by each beat and they decay exponentially between beats. The final"force of contraction" is the product of the two effects. The simulated curve for step frequency increase is dominated by the slow effect. The simulated curve for step reduction of frequency is dominated by the quick effect.

rested state contractions remain small, and in frequency jumps force rises and falls very quickly according to the quick phase with very little PIEA I in either the jump to a high frequency from the rested state or back to a low frequency (no curves in the interest of brevity).

It is not simple to account for the effects of ryanodine, even at a purely descriptive level. It was pointed out in chapter 4 that the normal mechanism of activation of rested state contractions in atrium seems to be independent of slow calcium inward current. Whatever that mechanism might be, ryanodine inhibits it in some way. As well, ryanodine strongly inhibits the normal positive inotropic effect of strong β-adrenergic stimulation on the rested state contractions (see 6.6). This last effect seems to be overcome quickly by increasing the contraction frequency, and figure 7.17.C presents a way of thinking about this effect, which is helpful:

The quick phase and the PIEA I, seen in frequency jumps after ryanodine treatment, must be related to one another in a special way, as reflected in the asymmetry of the force transients for increase and decrease of frequency. The effects seen in the jumps are <u>not</u> additive; the one depends on the other for its manifestation. This dependence is given by a multiplication of two cumulating effects, rather than an addition. The curve in panel C shows the kind of curve which results when two simple, linear effects are multiplied together; the simulated curve is not unlike the real frequency jump. According to the hypothesis that the PIEA I represents phosphorylation of slow calcium channels, the quick phase must represent an accumulation of calcium at a site from where it becomes available to the slow channels. The "multiplication" would correspond to the relationship between number of channels (=permeability) and calcium concentration available for production of slow inward current.

It is problematic to assess whether ryanodine has an effect on the rate of PIEA I decay. The slow phase of frequency inotropy decay <u>seems</u> to be accelerated somewhat

after ryanodine treatment, but this effect could be due to distortion by the calcium-force relationship, since the contractile level at 0.25 Hz is quite low. There clearly remains a phase of frequency inotropy decay after ryanodine treatment quicker than the PIEA I, but slower than the rapid phase of contractility loss seen after reduction of frequency (no curves presented). This is probably the PIEA II.

To summarize: The effects of ryanodine are more complex than any of the other interventions presented. 1) The mechanism of the normal rested state contractions in atrium is inhibited. 2) The mechanism by which ß-adrenergic stimulation increases rested states is inhibited (2 and 1 might be identical). 3) A quick phase of frequency inotropy accumulation and decay developes, which either does not normally exist or is not visible. 4) This quick phase is related to the PIEA I in the sense of a multiplication§.

§ The suggestion that the PIEA I and the quick phase could be related in this sense was made by Dr.Mensing after I puzzled for days about how the asymmetry described could come about.

7.5. Discussion.

It was pointed out in chapter 3 (figure 3.7) that the Bowditch part of frequency-force loops behaves very much as expected for a single, linear process. The present chapter unmasks a considerably greater deal of complexity. This is not necessarily an indication that the impression of simplicity was wrong. Rather, it may suggest that the processes differentiated in force transients are integrated in such a way that a single process, namely the PIEA I, determines for the most part the form of the Bowditch part of frequency-force loops. A second possibility to account for the complexity of force transients is that certain effects differentiated really are of only negligible importance in atrium, which does not exclude the possibility that they could be of more importance in other preparations. The approach taken in analysis of force transients was to assume that all differentiatable effects could be potentially important for an understanding of the underlying system. After appreciating this complexity it should be possible to fish out the processes of central importance in atrium.

The PIEA II could be reduced quite markedly with little change of either the PIEA I or the PIEA III. It does not seem to be of great importance. None-the-less, it would have been impossible to analyze the PIEA III without differentiating the PIEA II. The effect of dinitrophenol on the PIEA II is an indication that it could correspond to exchange of calcium to and from the mitochondria as this exchange influences the rest of the excitation-contraction coupling system. It is beyond question that mitochondria are capable of sequestering and releasing calcium, and their likely role in regulation of calcium is that of an intracellular calcium buffer (Bygrave, 1978). The mitochondria would sequester a small amount of the calcium activating each contraction. At the low intracellular calcium concentration maintained during diastole, the mitochondria would begin to release this calcium. Part of the calcium released by mitochondria between beats could become available for release at the next beat. Also,a release of calcium from the mitochondria between beats might in a still more

indirect way influence diastolic calcium exchange in the cell. Katzung et al. (1956) reported that dinitrophenol differentially suppresses post-stimulatory potentiation in atrium. It is certainly plausible that part of the slow part of PSP, taking seconds to develope, could result from a "transfer" of calcium from the mitochondria to sites from where it can be released for contraction.

The PIEA 0 also seems to be of no great importance to the normal production of the atrial frequency-force relationship. In frequency jumps it cannot even be differentiated. Circumstantial evidence supports the idea that the PIEA 0 could correspond to sodium exchange, coupled to an inotropic effect. Glitsch et al. (1973, 1978) presented evidence that either cooling or high-frequency stimulation lead to an intracellular acummulation of sodium. The result of such an accumulation is a transient hyperpolarization due to electrogenic pumping of sodium out of the cell by the sodium-potassium ATPase (3 Na^+ out/ 2 K^+ in). The time course of the membrane potential changes , which correspond to reestablishment of the normal intracellular sodium concentration, is very slow; 20 and more minutes are needed in guinea pig atrium to establish equilibrium. Also, the time course for sodium extrusion reported from direct ion measurements (Glitsch et al.,1970) was similarly slow. These findings support the idea that tha PIEA I cannot correspond to sodium accumulation; the time courses reported would correspond quite well to the time course for decay of PIEA 0. Finally, the slow phase of frequency inotropy, which developes after veratridine treatment, as well corresponds temporally to the PIEA 0.

The processes of central importance in atrium are obviously the PIEA I and the PIEA III (= NIEA at the rested state); a third process, present in frequency jumps but ignored by the form of analysis used, is responsible for the one-beat changes of contraction force with change of frequency (one-beat fall of force with step increase of frequency and one-beat rise of force with step decrease of frequency).

According to the hypothesis developed in previous chapters, the PIEA I decay would correspond to dephosphorylation of slow calcium channels. The temperature dependence of PIEA I decay would be expected for such a biochemical process. Also, the effects of ß-adrenergic stimulation on PIEA I decay may currently be explained in terms of a dephosphorylation. Phosphoprotein phosphatase activity may be inhibited parallel to stimulation of cAMP-protein kinase (Nimmo et al., 1978). Such a mechanism would serve to amplify the cAMP signal. The proposed mechanism would as well be sensible in that some ATP otherwise needed for phosphorylations could be saved. Carbachol would act by decreasing enhanced cAMP levels with a resulting increased phosphatase activity. Agents which do not affect the cAMP system would logically not affect the PIEA I decay rate.

The beat-dependence of certain inotropic effects of interval change has been described before. Morad and Goldman (1973) summarized the beat-dependent processes described to that time. In particular Wood et al. (1969, 1975) described such effects in ventricular muscle. Seibel (1975) analyzed beat-dependent inotropic effects in guinea pig papillary muscle. A straight-forward way of thinking about a beat-dependent kinetics is to consider it to correspond to a beat-dependent loss or gain of calcium in the electromechanical coupling system during a single contraction cycle. Morad and Goldman proposed that about 40% of the calcium activating contraction enters and leaves the cell in a single contraction cycle. In order to explain a beat-dependent kinetics it must be assumed that calcium <u>extrusion</u> as well as calcium <u>influx</u> is enhanced at each beat. Up to now the mechanism of calcium extrusion from the cell has received less attention than possible mechanisms for calcium influx. The beat-dependent effects of interval-change may be providing an important clue, namely that efflux of calcium from the electromechanical coupling system is beat-dependent. The central problem, however, is how and where a beat-dependent loss of calcium from the cell could take place.

For that matter, the whole question of how calcium is extruded from the heart cell is open to debate. Generally it has been assumed that sodium-calcium exchange would be responsible for calcium extrusion, although it is necessary to assume for this proposal that calcium accumulates in special places along the inside of the sarcolemma to concentrations considerably higher than in the cytosol (see Kaufmann et al., 1974). It is difficult enough to logically imagine how such a mechanism would function,without trying to explain why the mechanism would result in a beat-dependent calcium extrusion. An alternative hypothesis would be that a sarcolemmal calcium pump could compete with the sarcoplasmic reticulum at relaxation, and extrude a good deal of the calcium released at activation. At present, however, this possibility seems very unlikely; the calcium pump system of the sarcolemma shows a low maximal velocity of pumping (Caroni and Carafoli, 1980).

According to the above suggestion,as to how a beat-dependent kinetics could come about, the PIEA III factor would correspond to the fraction of calcium maintained in the electromechanical coupling system from one beat to the next. At the rested state contraction of guinea pig atrium (28°C) nearly 90% of the calcium in the system would be lost (PIEA III factor = 0.12). To date there seems to be no logical proposal to account for such a large beat-dependent calcium extrusion. Also, we need to look for a reason why the percent of calcium lost from the system should decrease with acummulation of PIEA I. No logical reason for this behavior arises out of the assumption that the calcium lost with each beat is lost either via calcium pumps in the sarcolemma or via sodium-calcium exchange. The hypothetical solution to this problem, proposed in the next chapter, would not only account for the apparently beat-dependent nature of calcium exchange to the electromechanical coupling system, but would also provide a new solution to the problem of how calcium is released from the sarcoplasmic reticulum.

7.6. Summary of chapter 7.

1) Four different processes can be shown to be involved in frequency inotropy decay in guinea pig atrium.

2) In force transients for reduction of frequency in a step two rapid phases of frequency inotropy decay (PIEA's II and III) and a predominant slow phase (PIEA I) are differentiated.

3) The PIEA's II and I decay with a given rate constant. The PIEA III decays with a strong dependence on the number of beats made.

4) The PIEA III corresponds to the same process responsible for loss of contractility upon stimulation after rested state contractions (= NIEA) and as well to the process decaying after single extrasystolic beats.

5) The kinetics of decay of the PIEA III depend on the amount of PIEA I present in a muscle preparation; decay is slower with more PIEA I.

6) The apparent amount of PIEA I produced per beat is <u>not</u> independent of the frequency at which the PIEA I is analyzed.

7) In rest decay curves a fourth process (PIEA 0) decays at a rate several times slower than the PIEA I, and this process cannot normally be differentiated in force transients for step reduction of frequency.

8) The PIEA II is specifically inhibited by dinitrophenol.

9) The PIEA I decay rate is strongly slowed by temperature reduction.

10) PIEA I decay rate is slowed by ß-adrenergic stimulation and can be accelerated by carbachol. It is not affected by the bath calcium concentration, by nifedipine or by heart glycosides.

11) Veratridine creates a new, slow phase of frequency inotropy acummulation and decay which shows a rate constant similar to the PIEA 0.

12) Ryanodine treatment results in a marked assymetry of frequency inotropy acummulation and decay. The rapid loss of contractility after cessation of electrical stimulation with ryanodine pretreatment does not correspond to decay of any of the above processes.

8. Simulated interval-strength relationships.

Chapter 8 is dedicated to the eminent scientific and ethical example set by VLADISLAF KRUTA, who died this year. Both during the years of fascism in Europe and in the years since 1968 in Czechoslovakia he placed his social responsibility above his career at a great personal risk.

"We do not lack experimental findings, there being perhaps too many, so that we cannot see them in their correct proportions, and find out the system behind them. Their interpretation is extremely difficult. (...) There is a need to attempt a synthesis, trying to trace the hidden connections and to identify the physicochemical elements of this important physiological mechanism."

--Vladislaf Kruta

at the close of the 1963 symposium on the interval-strength relationship in Prague§.

§ see Proceedings of the Second International Pharmacological Meeting, Vol. 5, 81-82, 1964.

8.1. A new model of excitation-contraction coupling.

In the introduction to this dissertation three questions were mentioned, which seem to sum up the problem of how membrane excitation is coupled to contraction: From where and how is calcium released for contraction activation? To where and how is calcium taken up for relaxation? How can these processes be efficiently controlled to regulate cardiac contractility? A solution to the puzzle of the interval-strength phenomena demands an answer to these questions. It would be an important test of any model of excitation-contraction coupling to show that it can deal with the phenomena of the interval-strength relationship. As well, the complexity of interval-strength phenemena makes it a considerable task just to be able to say, "this is the way it could be." This is the major purpose of this chapter.

As pointed out in the introduction, none of the simulated models of electromechanical coupling presented to date seem to be able to account for the atrial interval-strength relationship in a satisfactory way. This impression strengthened in my mind, during the course of the functional work presented, to the point that it seemed necessary to look for an alternative or modified scheme of how contraction activation takes place in heart. A viable alternative was given by the work of Mensing in this institute, who has proposed a model based on a critical evaluation of the literature of the field (1979). Although we have worked in close association over the last years, this model was developed independently from the functional findings presented. It was not pieced together s i m p l y to allow a simulation of the atrial interval-strength relationship, but rather in an attempt to come to terms with open questions of how contraction activation may function. My own reason for attempting a rough, digital simulation of this model was that I saw the possibility to explain a large number of interval-strength phenomena within its framework. The model of electromechanical coupling accepted here is in its conception a modified version of the scheme for calcium movements in mammalian myocardium proposed by Langer (1974). The informed reader will however

recognize parallels to other proposals as well. Nonetheless the present model certainly comes closest to that of Langer and associates in the question of the immediate source of activator calcium and the importance of the glycocalyx.

The model is diagrammed schematically at rest in the left panel of figure 8.1. No essentially new ideas are proposed. The heart cell is thought of as a two compartment system. The sarcolemma (glycocalyx plus unit membrane) divides off the cytosol with contractile proteins from the extracellular fluid. Within the cell the sarcoplasmic reticulum presents a second compartment, coupled in series with the cytosol to the extracellular fluid. In this well-accepted order of things, the calcium concentration of the cytosol at rest must be determined by the sarcolemma. The most important mechanism responsible for establishing the calcium gradient between the extracellular medium and the cytosol is considered to be the sodium-calcium exchanger. A ratio of $3Na^{+}$ to $1Ca^{++}$ is accepted for exchange; this ratio has been demonstrated recently on isolated sarcolemmal vesicles of heart (Pitts, 1979). For such an electrogenic ion exchange system, the coupling of the sodium gradient to the calcium gradient across the membrane will depend on the membrane potential. This coupling has been described by the following equation (see Baker, 1975):

$$(Na_i/Na_o)^3 \cdot e^{\frac{E_m \cdot F}{R \cdot T}} = (Ca_i/Ca_o),$$

It is well-accepted that the sodium-potassium pump can establish a sodium gradient across the sarcolemma in the range of 1 to 15. With this sodium gradient and an E_m of -70 mV the exchanger would maintain the intracellular calcium concentration at about 3×10^{-8} M with 2 mM calcium in the extracellular medium. This free intracellular calcium concentration would definitely be adaquate for maintaining relaxation. The intracellular sodium concentration would be one of the most important determinants of the intracellular calcium concentration. The sodium-calcium exchanger is considered to be capable of bidirectional ion exchange; a net influx or efflux of calcium could be produced by the exchanger under different cellular conditions.

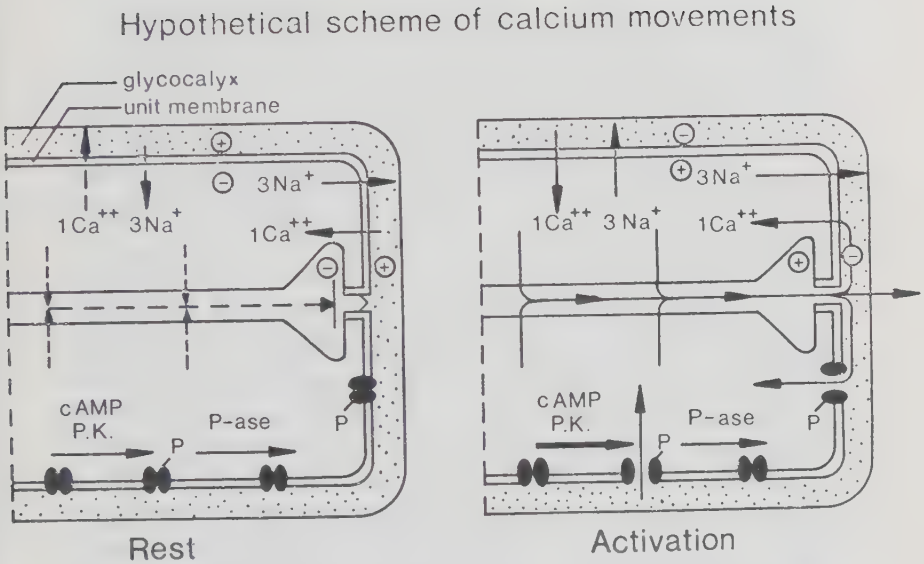


Figure 8.1. Essential elements of a new excitation-contraction coupling model. The bone-shaped structure inside the cell represents the sarcoplasmic reticulum with terminal cisternae and couplings to the sarcolemma.

cAMP P.K. = cAMP-dependent protein kinase. P-ase = phosphoprotein phosphatase. (●●) = slow calcium channel.

Two new mechanisms allow a simulation of the atrial interval-strength relationship: 1) Calcium is released from the terminal cisternae of the SR into the glycocalyx at depolarization, not directly into the cytosol. 2) Phosphorylation of slow calcium channels by cAMP-dependent protein kinase is facilitated at depolarization.

See text for further explanations of the schematic diagram and the excitation-contraction coupling model.

It is well-known that the sarcoplasmic reticulum is capable of rapidly sequestering calcium via an ATP-driven calcium pump. Therefore, this pump will establish a large calcium gradient between cytosol and the inside of the SR. Hasselbach (1980) estimates the potential calcium gradient across the SR membrane to be about 1 to 50,000.

At rest, slow calcium channels will be continually phosphorylated and dephosphorylated; the equilibrium between phosphorylated and unphosphorylated channels will depend on the activity of cAMP-dependent protein kinase and phosphoprotein phosphatase. It is assumed that only phosphorylated slow calcium channels will open at depolarization.

The right panel of figure 8.1 diagrams the situation in this model at depolarization. The existence of special calcium pores or channels is postulated in the couplings between SR and the sarcolemma. These channels, as other channels, would be too small to be detected electromicrospically. The channels would experience the membrane potential of the sarcolemma and would open at depolarization. Under the assumption that the SR membrane is permeable to cations other than calcium, the+30 to 40 mV action potential overshoot would produce an electrical gradient for calcium to leave the terminal cisternae of the SR. If the free calcium concentration of the SR is roughly the same as the extracellular concentration, an approximately 1 to 10 electrochemical gradient for calcium to leave the SR would be established. The calcium released from the SR in this fashion would not be immediately lost from the cell; the glycocalyx surrounding the cell would serve as a diffusion barrier, so that time enough would be given for part of the calcium released from the SR to reenter the cell. The function of the glycocalyx as diffusion barrier, in part due to its large calcium binding capacity, would result in a higher calcium concentration in the glycocalyx nearer to the SR-sarcolemmal couplings than at points farther away for 10 to 30 ms.

Activation of contraction would result from calcium entrance into the cell by two mechanisms at depolarization.

First, phosphorylated slow calcium channels are opened by depolarization. Second, the equilibrium for sodium-calcium exchange is shifted by depolarization to +30 or 40 mV to 10^{-6}M intracellular calcium and more, according to the above equation; the "more" refers to the areas surrounding SR-sarcolemma couplings where the free calcium concentrtion is higher than the free extracellular concentration. As a result, calcium would also enter the cell via sodium-calcium exchange. Most calcium entering the cell via sodium-calcium exchange would be of SR origin. As well, some of the SR calcium released would reenter the cell via slow calcium channels. A third portion of the SR calcium released would be lost from the cell with each beat. Calcium entering the cell via slow channels and sodium-calcium exchange would together be enough for activation of contraction. The postulation of calcium channels in the couplings of the SR to sarcolemma would solve both the problem of how calcium is released from the SR, where it is stored, and how calcium leaves the cell.

By far the greatest part of calcium activating contraction would be taken up by the SR for relaxation. A small portion of activator calcium might be lost from the cell with each beat via a sarcolemmal calcium pump or via sodium-calcium exchange after repolarization.

Finally, for reasons dealt with in detail in previous chapters it is assumed that with each depolarization a small per cent of the unphosphorylated slow calcium channels are phosphorylated by cAMP-dependent protein kinase.

The parallels of this model to the "one-way" Langer model for calcium movements are:1) All calcium for activation is considered to enter the cell with each beat via sodium-calcium exchange and slow calcium channels. 2)Calcium movement is unidirectional. 3)The glycocalyx is considered to play an important role in regulation of contractility. A parallel to considerations made by Reiter (1966) is that calcium would leave the cell only at special locations--in contrast to sodium--and that under certain circumstances the calcium concentration at such

extracellular sites would be an important determinant of calcium influx/beat. A precedent for a voltage-dependent release of calcium from the terminal cisternae of the SR to the glycocalyx is present in the model of Kaufmann et al. Although this extrusion mechanism apparently is responsible for the elimination of only relatively small amounts of calcium, its postulation can be assumed to be important for the accurate simulation of interval-strength phenomena in kitten papillary muscle (1974).

8.2. General description of the simulation program.

The program for discrete, "beat-to-beat" simulation of the excitation-contraction coupling model outlined above is printed in 8.5 with explanations. Three steps are involved in the calculation of"peak contraction force of each beat": 1) The amount of calcium available for release from the sarcoplasmic reticulum is calculated, as determined by the calcium taken up by the SR at the previous beat and the interval to the present beat. 2) The fraction of the calcium released is calculated which will reenter the cell. 3) The amount of calcium entering the cell of extracellular origin is calculated. The final amount of activator calcium is related to force of contraction via a sigmoid function (= calcium-force relationship). For all curves presented it is assumed that all activator calcium is taken up by the sarcoplasmic reticulum for relaxation.

8.2.1. Calculation of SR-calcium for release at each beat.

The amount of calcium present in the SR after an individual beat is the sum of SR-calcium which was not released at that beat and the amount of calcium which just activated that beat. In this simulated model the assumption that up to 15% of activator calcium is not taken up by the SR results in only small changes. For simplification, therefore, it is assumed that all calcium activating a contraction is taken up by the SR, and that calcium extrusion at the sarcolemma is zero.

Calcium entering the SR is not immediately available for release. It is simply assumed that calcium entering the SR becomes available for release exponentially. Half-lives of not less than 300 ms and not more than 1500 ms

must be assumed to give a realistic simulation. The relative slowness of this process may reflect a diffusion resistance in the SR, for example calcium-binding proteins.

Between beats the calcium fill level of the SR approaches a rest equilibrium. It is assumed that the SR membrane, itself, is completely impermeable to calcium, and that calcium exchange to the cytosol takes places solely via the calcium pump. The rate of calcium exchange from the SR to the cytosol (k_2) would correspond to the rate at which the calcium pump runs backward, a process which has been well documented (Hasselbach, 1980). A stoichiometric ratio of 2 mol calcium pumped per 1 mol ATP is accepted (Hasselbach, 1980), which results in a second order kinetics for calcium exchange:

$$\frac{dCa_{SR}}{dt} = k_1(Ca)^2_{cytosol} - k_2(Ca)^2_{SR}.$$

Assuming that the cytosol calcium concentration is effectively held constant at rest by the sodium-calcium exchanger, the equation simplifies to:

$$\frac{dCa_{SR}}{dt} = K - k_2(Ca)^2_{SR}.$$

The simplified equation would be an approximation of calcium exchange with the SR for rest only; for relaxation the assumption is made that the high k_1 of the SR in relation to other calcium-pumping systems (mitochondria, sarcolemmal calcium pump) results in the sequestering of nearly all activator calcium by the SR. The greatest portion of activator calcium would be pumped into the SR before the membrane potential returns to a level favoring calcium extrusion via sodium-calcium exchange. These are of course oversimplifications, but as pointed out above, the assumption that small portions of activator calcium are not taken up by the SR results in little change of the simulated phenomena.

The simplified equation gives curves for the approach to equilibrium of SR-calcium at rest as presented in figure 8.2. In the left panel of the figure curves are given

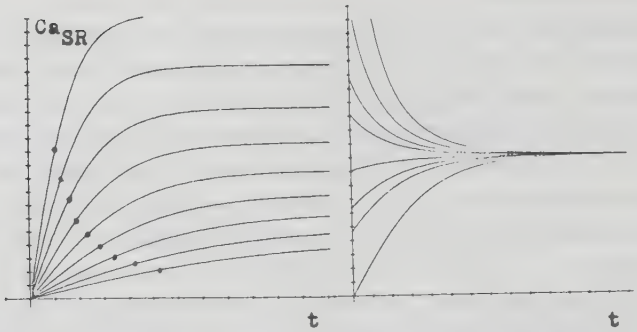


Figure 8.2. Curves for return to equilibrium of the calcium fill level of SR according to the simplified differential equation. Left panel: curves for return to equilibrium from zero with various cytosolic calcium concentrations. Dots mark the 50% fill levels for the curves. Right panel: Curves for return to equilibrium from various starting levels, but the same cytosolic calcium concentration. Curves were made by calculating point-to-point with the differential equation.

for return to equilibrium from zero calcium level in the SR, but with various hypothetical cytosolic calcium concentrations. Note that the point at which 50% of equilibrium is reached, marked by dots, varies with the final fill level and is determined by the cytosolic calcium concentration. In the right panel of the figure curves are given for return to equilibrium from various SR fill levels at a constant cytosolic calcium concentration.

To describe this process in the simulation program the following equation system was derived from the differential equation and was checked by comparison to curves as in figure 8.2:

Ca_{SRo} = calcium fill level at t_o
Ca_{SRt} = calcium fill level at t (= beat interval)
Ca_{SRe} = calcium fill level at equilibrium (infinite t)
K_{SR} = rate constant (not identical with k_2 above).

$$B = \frac{(Ca_{SRo} + Ca_{SRe})}{|Ca_{SRo} - Ca_{SRe}|}$$

for $Ca_{SRo} > Ca_{SRe}$, $Ca_{SRt} = Ca_{SRe} \cdot \frac{B + e^{-K_{SR} \cdot t}}{B - e^{-K_{SR} \cdot t}}$

for $Ca_{SRo} < Ca_{SRe}$, $Ca_{SRt} = Ca_{SRe} \cdot \frac{B - e^{-K_{SR} \cdot t}}{B + e^{-K_{SR} \cdot t}}$

The K_{SR} in the equation system is not identical to the k_2 in the differential equation; curve form produced by the equation system is however identical to curves as in figure 8.2. In simulated curves the diastolic calcium concentration of the cytosol is considered to remain constant under given conditions (constant Ca_{SRe} and K_{SR}). For any intervention thought to logically increase or decrease the cytosolic calcium concentration at rest, the Ca_{SRe} and the K_{SR} are changed in parallel to duplicate the curves given by the differential equation.

It is assumed that not all calcium in the SR is potentially releasable. A parameter must be set which gives the fraction of SR calcium which will be released after time enough for diffusion and calcium exchange in the SR to reach equilibrium. This gives the fraction of SR calcium released to the glycocalyx at the rested state. At higher frequencies it gives the fraction of SR calcium which potentially can diffuse into the terminal cisternae and be released. To give realistic simulated frequency jumps it must be assumed that at least 70% of the total SR calcium is released at the rested state. For all simulations presented it is assumed that 75 or 80% of the SR calcium is released at the rested state. This emperically set parameter would indicate that after rest most SR calcium is located in the terminal cisternae.

Finally, it is assumed that all potentially releasable calcium in the terminal cisternae is actually released to the glycocalyx at depolarization. More complicated assumptions about the function of the SR can be made to give a more exact simulation, but the present chapter is intended to show how the <u>simplest</u> assumptions can account for a large number of interval-strength phenomena in a quite realistic way.

8.2.2. Simulation of slow calcium channels.

The number of phosphorylated calcium channels available for opening at depolarization is represented in the program by a fraction from 0 to 1. A parameter must be set

to give the fraction of slow calcium channels phosphorylated at rest. A second parameter is set, which gives the fraction of unphosphorylated channels, phosphorylated with each beat according to the hypothesis that the approach of catalytic subunit of cAMP-protein-kinase to the inside of the sarcolemma is facilitated at depolarization. For any interventions thought to change the activity of cAMP-protein-kinase the parameters for rest phosphorylation and phosphorylation per beat are changed in parallel. Finally, dephosphorylation of slow channels is assumed to be a simple, exponential process.

The fraction of phosphorylated slow calcium channels is used for two calculations: 1) The hypothetical amount of calcium of extracellular origin entering the cell at each beat is assumed to be proportional to the fraction of phosphorylated slow channels. A parameter must be set which gives the amount of extracellular calcium entering the cell via slow channels at 100% phosphorylation. 2) The fraction of SR-calcium released which is taken up again via slow calcium channels is also considered to be proportional to the fraction of slow channels phosphorylated. A parameter must be set which gives the fraction of SR-calcium released, which is taken up again via slow calcium channels at 100% phosphorylation.

8.2.3. Simulation of sodium-calcium exchange.

For most simulated curves presented it is assumed that the intracellular sodium concentration remains constant with change of frequency. Under these conditions it is simply assumed that a certain, relatively small, amount of calcium enters the cell with each beat via sodium-calcium exchange from the extracellular fluid. Also, a certain, constant fraction of SR-calcium released is assumed to reenter the cell via sodium-calcium exchange. A crude simulation of how the intracellular sodium concentration could behave with frequency change is presented in 8.4.

8.2.4. Summary of the simulation parameters.

The final simulation system has 11 possible variables. The variables cannot however be considered independent, and there are logically only 3 or 4 ways to manipulate or influence the system. The parameters corresponding to the activity of the cAMP system can be increased or decreased. The slow inward current can be increased or decreased independent of the activity of the cAMP system. For any interventions which would logically influence the diastolic calcium concentration, the equilibrium level of the SR and its rate constant should be changed in parallel. The variables for rate of diffusion in the SR and the fraction of releasable SR-calcium actually released would logically remain constant for all interventions simulated.

1) Ca_{SRe} = fill level of the SR at rest.
2) K_{SR} = rate constant for the approach to equilibrium of the SR-calcium fill level between beats.
3) K_d = rate constant for passive movement of SR-calcium from uptake sites to release sites.
4) F_{SR} = fraction of SR calcium potentially available for diffusion to release sites.
5) Ca_x = Amount of calcium of extracellular origin entering the cell via sodium-calcium exchange at each beat.
6) Ca_p = Amount of calcium of extracellular origin which would enter the cell via slow calcium channels at 100% phosphorylation level.
7) F_x = Fraction of released SR-calcium which reenters the cell via sodium-calcium exchange at each beat.
8) F_p = Fraction of released SR-calcium which would reenter the cell via slow calcium channels at 100% phosphorylation level.
9) F_r = Fraction of slow calcium channels phosphorylated at the rested state.
10) F_{PIEA} = Fraction of unphosphorylated slow calcium channels which are phosphorylated with each beat.
11) K_{PIEA} = Rate constant for dephosphorylation of slow calcium channels.

8.2.5. Setting the simulation parameters.

The three rate constants needed for simulation are taken from functional experiments. K_{SR} corresponds to the slow part of the NIEA decay curve according to the Blinks/Koch-Weser method (to be described). K_d corresponds to the quick restitution of contractility as determined by the method of Kruta above 0.5 Hz. K_{PIEA} corresponds to the rate of PIEA I decay as described in the preceding chapter. F_x and F_p can be estimated from a frequency jump series and correspond to the PIEA III decay factor at the rested state and immediately after high-frequency stimulation in the presense of B-adrenergica, respectively. F_r is estimated from the size of the negative inotropic effect of strong purinergic stimulation or high concentrations of nifedipine on the rested state contractions under given conditions. Changes of F_r and F_x with B-adrenergic stimulation are estimated from the size of the positive inotropic effect at the rested state. All "Ca" values are arbitrary; intermediate "force" values result on a sigmoid function (calcium-force relationship) with a maximum of 100 and an EC_{50} of 50 (see 7.2; $EC_{50}=K$). A Hill coefficient for the calcium-force relationship of 4 is used in all simulated curves presented; a coefficient of 5 is too large and 3 toosmall to accurately reproduce distortions from linearity in the extreme ranges of contractility found in frequency-force loops (see the lower part of frequency-force loops in figure 8.8; curves 1; to be described).

8.3. Simulated curves.

8.3.1. Simulated frequency-force loop.

The simulated frequency-force loop presented in figure 8.3 is produced with parameters set to correspond to an atrium at 32°C, 2.5 mM calcium. An accurate judgement of the degree to which this curve form corresponds to the form of real frequency-force loops is difficult, since the frequency generator used in experiments does not produce a perfect exponential frequency change (see figures 3.6 and 3.7). The rate of frequency change chosen for simulation

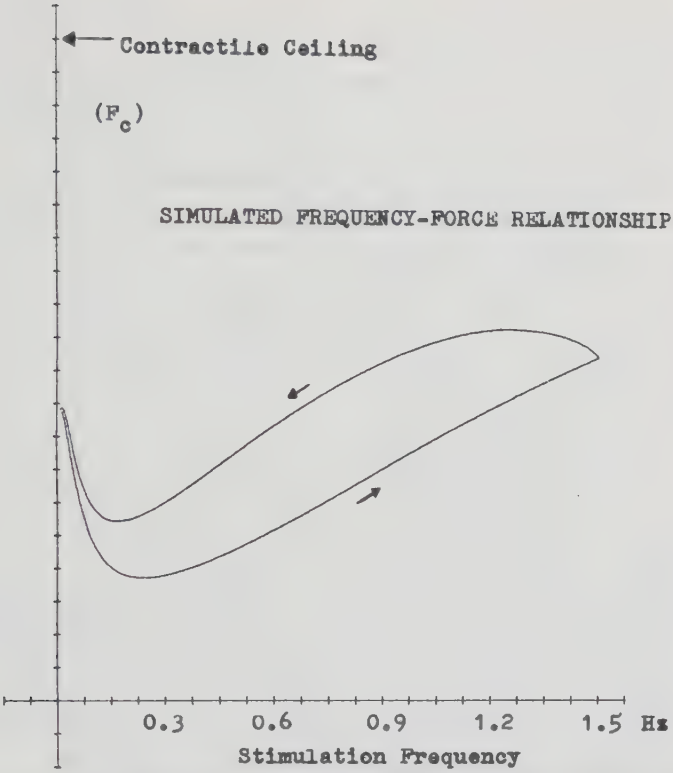


Figure 8.3. Simulated frequency-force relationship with parameters set to correspond to an atrium at 32°C, 2.5 mM calcium. Exponential frequency change is set at the average rate made by the frequency generator. To the parameter settings: Hypothetical calcium amounts and force amounts are arbitrary. Calcium amounts are set to give intermediate force values on a sigmoid function (calcium-force relationship) with a force maximum of 100, a calcium EC_{50} of 50 and a Hill coefficient of 4. See text for further explanations.

Parameter settings:

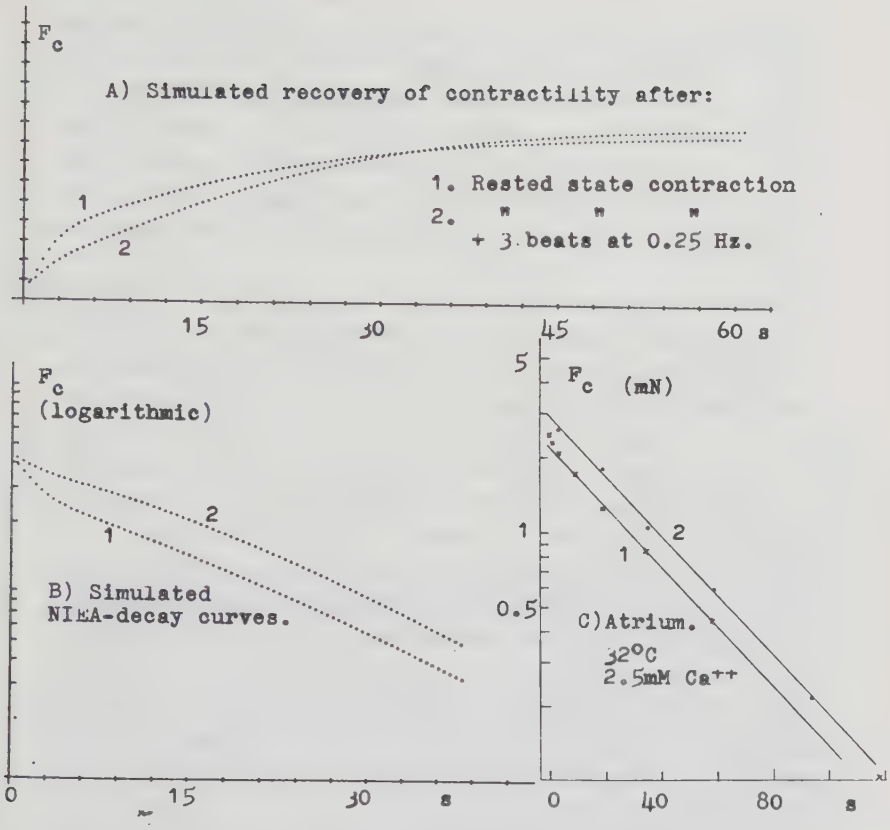
1) $Ca_{SRe} = 200$
2) $K_{SR} = 0.06s^{-1}$
3) $K_d = 0.75s^{-1}$
4) $F_{SR} = 0.8$
5) $Ca_x = 16$
6) $Ca_p = 30$
7) $F_x = 0.15$
8) $F_p = 0.5$
9) $F_r = 0.07$
10) $F_{PIEA} = 0.009$
11) $K_{PIEA} = 0.014s^{-1}$

corresponds to the average rate of frequency change made by the frequency generator (t_{50}= 170 s). One real and obvious deviation of simulated curves from real curves is that the rising and falling parts of the frequency-force loop do not cross in the simulated curve at a low frequency. This deviation will be considered again later and a possible correction will be proposed to make this aspect of the simulated curves more realistic. The minimal simulation program used here is enough to illustrate how the proposed electromechanical coupling model can account for a considerable number of interval-strength phenomena and pharmacological interventions.

8.3.2. NIEA decay curves.

Blinks and Koch-Weser (1961) analyzed the effect responsible for loss of contractility in the Kruta part of the atrial frequency-force relationship by following the recovery of contractility after single rested state contractions ("NIEA decay curves"). Usually it takes a minute and more for a preparation to regain full rested state contractility. Obviously, this slow process of recovery does not represent the same process analyzed by Kruta and coworkers at higher frequencies, where contractility recovers for the most part within 1 or 2 seconds.

Figure 8.4 presents NIEA decay curves from the simulated model and a real atrium: In figure 8.4.A the strength of test contractions at various intervals after a rested state contraction is plotted linearly against time (simulated curves with parameters set as in 8.3.). Curve 1 is for recovery of contractility after a single rested state contraction, the normal Blinks/Koch-Weser method. In panel B, curve 1 of the figure, this curve is plotted semi-logarithmically after subtraction of the assymptote line. The resulting NIEA decay curve shows a rapid and a slow part in the simulated model, as does a real atrium (panel C, curve 1). This is also typical for curves presented by Blinks and Koch-Weser. According to the model proposed here, the quick part of this curve corresponds to



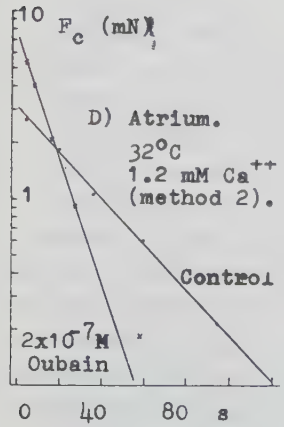


Figure 8.4. NIEA decay curves.

Panel A shows recovery of contractility after single rested state contractions (1) and after rested state contractions followed by 3 beats at 0.25 Hz (2) in simulated curves. Panel B shows the resulting NIEA decay curves after subtraction of an assymptote and plotting in a logarithmic force scale. Panel C show corresponding curves from a real atrium. In model and atrium the slow part of the curve is enhanced at the cost of the rapid part after 3 beats at 0.25 Hz. Panel D demonstrates the effect of an intermediate concentration of oubain on the slow part of NIEA decay, determined by method 2. Decay rate is accelerated and the intercept is increased.

the movement of calcium taken up at the rested state contraction from uptake to release sites. Since a large portion of calcium released to the glycocalyx at the rested state beat is lost to the extracellular bulk, a quite large calcium deficit in the SR is produced by the rested state beat. This deficit is then corrected slowly according to the constant for return to equilibrium of the SR-fill level. This process corresponds to the slow part of the NIEA decay curve.

Curves 2 in panels A, B and C show NIEA decay as depicted by a different method in the simulated model (A and B) and from a real atrium (C). After a rested state contraction under the conditions of figure 8.4, the fill level of the SR is reduced to about 30% of its equilibrium level. If a second beat is made after completion of calcium diffusion from uptake sites to release sites, but before the fill level has markedly returned toward equalibrium, the fill level of the SR can be reduced still further. After 3 beats at 0.25 Hz the NIEA decay curve in model and atrium shows only the presence of the slow part. The slow phase becomes larger at the cost of the quick phase. This phenomenon, which at least appears very similar in model and atrium, was predicted by the model before the atrium curves were made. The phenomenon was described in part by Blinks and Koch-Weser (1961).

According to the model the intercept of NIEA curves made by method 2, recovery of contractility after 3 beats at 0.25 Hz, will r e f lect the SR-fill level at the rested state. Panel D of figure 8.4 shows the typical effect of heart glycosides on the NIEA decay curve as depicted by this method. The intercept of the curve is greatly increased and the rate of NIEA decay is strongly accelerated. According to the model (see figure 8.2) an increase of the intercept and an acceleration of the decay rate will always go hand-in-hand. An increase of intracellular sodium would lead to an increase of the intracellular calcium concentration and thereby to an increased SR-fill level. Increasing the bath calcium leads to very similar effects on the NIEA curves made with this method (no curves presented).

Force Transients in Response to Frequency Reduction.

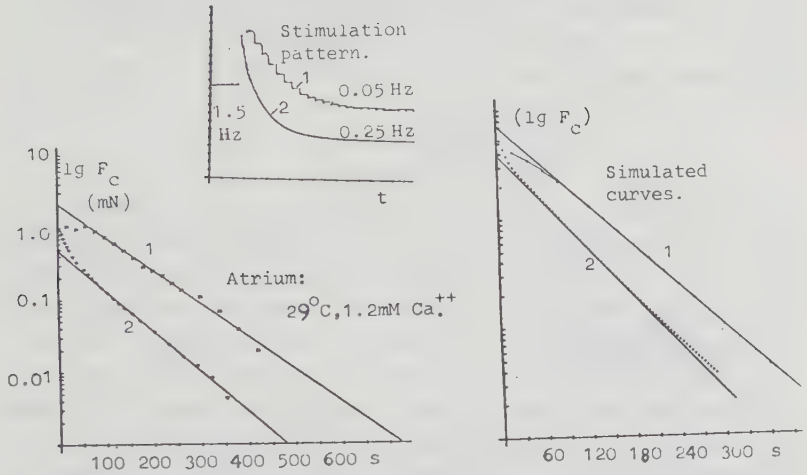


Figure 8.5. Comparison of frequency jumps from 1.5 to 0.25 Hz and from 1.5 to 0.05 Hz in atrium and model. The dots in the simulated curves correspond to individual beats. See text for further explanations.

8.3.3. Force transients for frequency reduction.

Figure 8.5 presents the model's response to step reduction of frequency as analyzed in chapter 7. The size of the PIEA I intercept is greater for a jump from 1.5 to 0.05 Hz than for the jump from 1.5 to 0.25 Hz, greater than predicted by the absolute frequency differencies. This comes about because the SR has time to refill toward equilibrium at the lower frequency. The extra calcium results in an amplification of the PIEA I decay line. The PIEA I decay line at 0.25 Hz appears to be slightly accelerated in relation to the PIEA I at 0.05 Hz (representative for 5 similar comparisons). The acceleration is <u>not</u> due to the calcium-force relationship. It comes about because the SR calcium fill level continually is decreasing at 0.25 Hz, parallel to dephosphorylation of slow calcium channels. The apparent acceleration of PIEA I decay at 0.25 Hz can be made understandable by considering what happens when two processes, related to each other in the sense of a multipication, decay with the same rate constant; in this case, the two processes

would be the calcium in the SR which becomes available for entrence into the cell via the slow calcium channels and the permeability of the slow channels as determined by their phosphorylation state. Consider the following two number series, and what happens when they are multiplied together:

1 x 80 = 80 (a
0.5x 40 = 20 (b
0.25x 20 = 5 (c
0.12x 10 = 0.125 (d

Let the series 1 to 0.12 in a to d be a measure for the degree of phosphorylation of slow channels, and the series 80 to 10 in a to d be a measure of the calcium available for release from the SR, which will decrease parallel to dephosphorylation of slow channels in this model. Each step in the number series from a to d will be one t_{50} for the dephosphorylation and the calcium fill level. If these two processes are multiplied, the resulting values go through <u>two</u> t_{50}'s in each step of the series. The slow inward current in the model results from such a multiplication, although not only from such a multiplication. As a result of this relationship, it must now be concluded that the rate constants determined for PIEA I decay in frequency jumps from 1.0 to 0.25 Hz represent a 10 to 25% overestimation of the rate of dephosphorylation. The decay curve at 0.05 Hz is not subject to this distortion, since the calcium fill level returns quite a ways back to equilibrium between beats. Note: This distortion of the real PIEA I decay rate in frequency jumps from 1 to .25 Hz may <u>in part</u> explain the slowing of PIEA I decay by β-adrenergic stimulation described in chapter 7.

The jump from 1.5 to 0.25 Hz begins with a force overshoot, and approaches the PIEA I decay line in 6 to 10 beats. The force overshoot corresponds to diffusion of calcium backed up in the SR to the terminal cisternae; the rapid decay phase corresponds to the calcium recirculation cycles necessary to bring the SR level down to the level of equilibrium given by the lower frequency and phosphorylation level. The first beats in the frequency jump to 0.05 Hz fall below the PIEA I decay line; in these beats the SR is actually filling <u>upward</u> toward the high fill level given by the low frequency (in atrium).

"...the earlier an extra contraction comes, the stronger is the following contraction;...two extra contractions are apt to be followed by exceptionally strengthened beats."

--Woodworth, 1902.

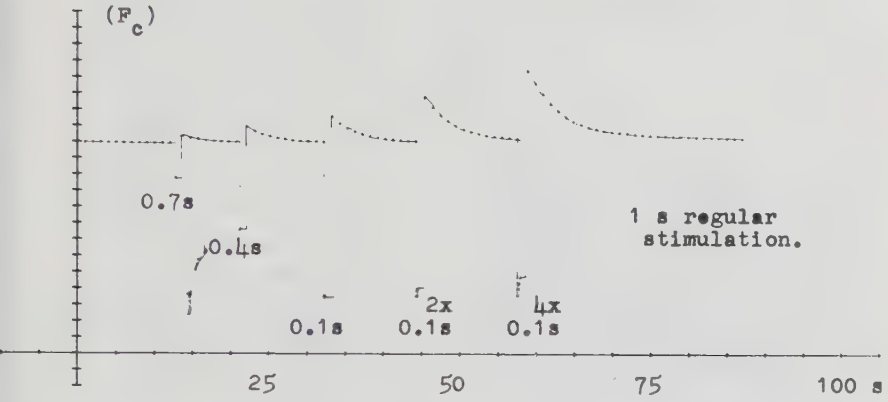


Figure 8.6. The simulated effect of extrasystolic beats. During a regular train of "impuses" at 1 Hz, extra beats are added at various intervals after a regular beat, and stimulation is continued at the regular 1 s interval.

8.3.4. Extrasystolic beats.

The effect of extrasystolic beats in the simulated model is also quite realistic. Figure 8.6 demonstrates the effect of adding single and multiple extra beats in a train of regular beats at 1 Hz; parameter settings are as in figure 8.3. As is well-known, the positive inotropic effect of an extra beat is greater, the smaller the size of the extra beat. This comes about in the model because calcium movement from uptake sites to release sites in the SR is not completed at intervals less than about 2 s. At the extra beat, even at the small intervals where contraction force is small, a considerable amount of calcium still enters the cell, but due to the lower non-linear part of the force-calcium relationship this calcium does not produce a very large beat. With the next beat at the regular interval part of this calcium and the calcium not released

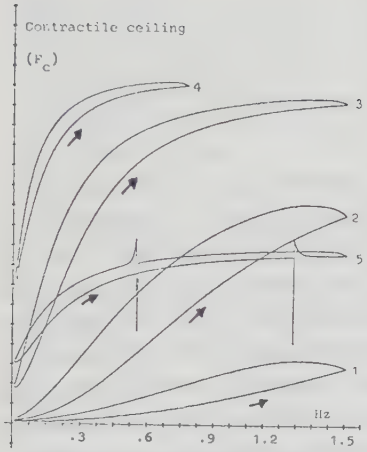
at the extra beat is available for release, and produces the potentiation of the following beats. This effect comes about essentially in the same way as in the model of Kaufmann et al. (1974), although the mechanism of calcium release from the SR is different in their model.

8.3.5. Simulated effects of stimulation of the cAMP system and reduction of slow inward current on the Bowditch part of the frequency-force relationship.

For a simulation of the frequency-force loops found at 28°C/1.2 mM calcium (after a short orciprenaline treatment) the following parameter adjustments must be made: The rate constant and equilibrium level of the SR are reduced. The PIEA rate constant is reduced. The PIEA per beat is increased somewhat. The amount of calcium entering the cell per beat via sodium-calcium exchange from the extracellular fluid and from the SR is reduced.

The figure 8.7 presents simulated curves under these conditions for interactions of B-adrenergic stimulation with nifedipine or adenosine (=ATP) as they would logically occur in the simulated system. From curves 1 to 2, 2 to 3 and 3 to 4 the rest phosphorylation level and the phosphorylation per beat were increased in parallel with no changes of other parameter. Note: Curve 2 would correspond to a control curve at 28°C and 1.2 mM calcium. The difference between curves 2 and 1 corresponds well to the effect of carbachol under these conditions; for adenosine or nifedipine the small bowing of the control curve from linearity in the high frequency range would be maintained (no curves presented).

From curve 4, with a quite stimulated protein kinase activity, to curve 5 only the factors translating phosphorylated slow channels into slow inward current were reduced (Ca_p and F_p). Two extrasystolic beats were added in the curve 5 to demonstrate that the mechanism responsible for this effect is still intact, although the mechanism of frequency inotropy production is exhausted at frequencies above about 0.3 Hz. In this simulated model, contraction force does not increase with increase of frequency unless either slow inward current or the



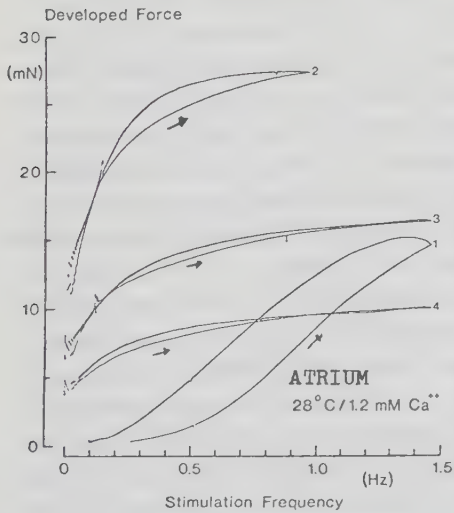
Simulated curves. Two extrasystolic beats were simulated in curve 5.

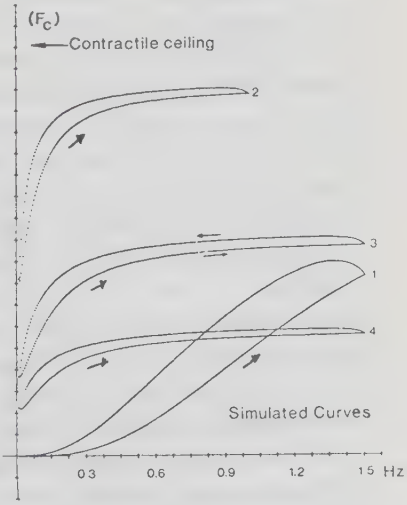
Figure 8.7. Simulated curves with parameters set to correspond to atria at 28°C, 1.2 mM Ca^{++}.

Parameter settings for curve 2:

1) $Ca_{SRe} = 60$
2) $K_{SR} = 0.02s^{-1}$
3) $K_d = 0.75s^{-1}$
4) $F_{SR} = 0.8$
5) $Ca_x = 8$
6) $Ca_p = 30$
7) $F_x = 0.07$
8) $F_p = 0.5$
9) $F_r = 0.1$
10) $K_{PIEA} = 0.02$
11) $K_{PIEA} = 0.01s^{-1}$

For the curves 1 to 2 to 3 to 4 the F_r and F_{PIEA} were increased in parallel. From curve 4 to curve 5 the Ca_p and F_p were reduced in parallel with no other parameter changes. Compare these curves to interactions of orciprenaline and nifedipine (figure 6.3). See text for further explanations.





1) control
2) 1.6 x 10^{-6}M orciprenaline
3) " " " + 3x10^{-5}M ATP
4) " " " + 10^{-4}M ATP

1 to 2) Increase of F_{PIEA} and F_r; and reduction of K_{PIEA}

2 to 3 and 3 to 4) reduction of Ca_p and F_p.

Figure 8.8 Interactions of orciprenaline and purinergic stimulation in model atrium. See text for further explanation.

intracellular sodium concentration increases. Only under special conditions would calcium in the SR accumulate alone and produce frequency inotropy (to be discussed). Compare curves in 8.7 with nifedipine-orciprenaline interactions described in figure 6.3.

In figure 8.8 the simulation parameters were set to correspond as exactly as possible to an atrium for interactions of orciprenaline and ATP (28°C; 1.2 mM Ca^{++}). From curve 1 to 2 only the simulated cAMP system was stimulated; in this case the rate of dephosphorylation was slowed along with an increase of the rest phosphorylation and the phosphorylation per beat. These adjustments correspond to the behavior of force transients analyzed and would indicate that stimulation of protein kinase is supported by an inhibition of phosphoprotein phosphatase as amplification mechanism. From curve 2 to 3 and 3 to 4 only the factors for translation of phosphorylated channels into slow inward current were reduced (Ca_p and F_p) in parallel. Note: For nifedipine and purinergic stimulation the maximal slow inward current of extracellular origin (Ca_p) and the maximal fraction of released SR calcium taken up via slow channels (F_p) are reduced in parallel, although marked differences in curve form do not result if these parameters are reduced to different extents. For a reduction of the extracellular calcium the Ca_p would logically be reduced more than the F_p; the fraction of released calcium taken up via slow channels would theoretically be little changed by a reduction of calcium, itself. This would explain why in figure 7.12 the quick phase of frequency inotropy remains slowed after ß-adrenergic stimulation is antagonized by reduction of the bath calcium. ß-adrenergic stimulation increases the fraction of calcium from the SR taken up via slow channels and which determines part of the quick phase of frequency inotropy decay (no change of F_p, but of the rIEA III decay factor as determined by the level of phosphorylation).

Note the bowing of frequency-force loops in the low frequency range under control conditions (curves 1). This

bowing is a reflection of the non-linear, lower part of the calcium-force relationship. It is described well with a Hill coefficient of 4 for the calcium-force relationship. Under some conditions (no curves presented) a bowing of curves in the upper force range also probably reflects the shape of the calcium-force relationship, and again, a Hill coefficient of at least 3 is needed to describe the bowing.

8.3.6. Simulated interactions of adenosine with orciprenaline and increased bath calcium at 32°C.

Figure 8.9 illustrates how simulating a certain effect can lead to a new insight. The abolition of the normal frequency-force relationship by the interaction of orciprenaline and adenosine (see chapter 3) cannot be completely explained by an increased phosphorylation of slow calcium channels due to ß-adrenergic stimulation and a cAMP-independent reduction of slow inward current by adenosine. If for example parameter settings are taken as in figure 8.3 (32°C; 2.5 mM calcium), a stimulation of the phosphorylation parameter and reduction of the slow inward current parameter results in a frequency-force relationship with an overly-large Kruta part for the adenosine-orciprenaline interaction. For a relatively realistic simulation of this interaction it must be assumed that the rest fill level of the SR and its rate constant for refilling are reduced. That is, it must be assumed that for this interaction the cytosolic calcium concentration is in some way reduced at rest in comparison to control conditions.

For the curve 1 in figure 8.9 parameters were set to give a roughly realistic frequency-force relationship for the adenosine-orciprenaline interaction at 32°C (compare with curves in figures 4.2 and 4.3). Changes of only the parameters for slow inward current independent of the cAMP system (Ca_p and F_p) result in the usual parallel upward and downward shifts of the horizontal frequency-force relationship (curve 1 to curve 2 for addition of more adenosine; curve 1 to curve 3 for removal of adenosine). From curve 3 to curve 4 the enhanced phosphorylation parameters were reduced with, again, no other parameter changes. Note that the control frequency-force relationship shows a much too small Kruta part to be realistic. It must be assumed that ß-adrenergic stimulation results in a reduction of the cy-

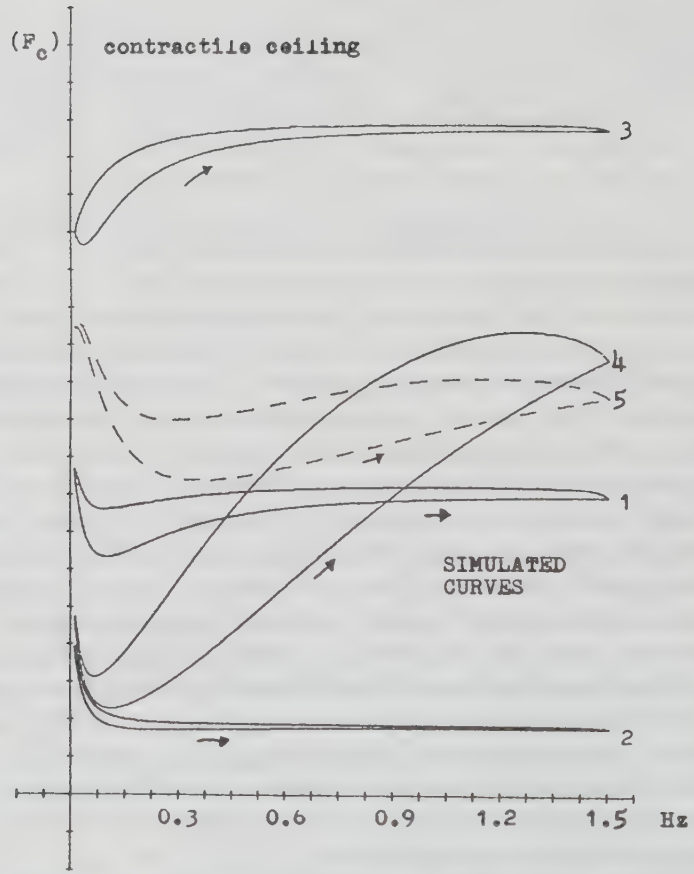


Figure 8.9. For curve 1, parameters were set to give the basic adenosine-orciprenaline interaction at 32°C. Parallel reduction or increase of the parameters for slow inward current result in the normal upward and downward shifts of the horizontal frequency-force relationship found with changes of the adenosine concentration (curves 1 to 2 and 1 to 3). From curve 3 to curve 4 only the phosphorylation parameters were reduced (= orciprenaline wash-out). Note that the curve 4 with SR-parameters set for the adenosine-orciprenaline interaction is unrealistic; the Kruta part of the relationship is much too small. For curve 5 in the figure the rest fill level of the SR and its fill rate were increased; slow inward current parameters are set 20% below the level in curve 4, calcium influx via sodium-calcium exchange is increased. These parameter settings would correspond to high bath calcium antagonized by adenosine. See text for further explanations.

tosolic calcium concentration.

A logical basis for this additional effect of ß-adrenergic stimulation is the hypothesis that sodium-potassium ATPase is subject to stimulation by a cAMP-dependent phosphorylation mechanism. Such a mechanism would result in the reduction of the intracellular sodium concentration and via sodium-calcium exchange in the reduction of the intracellular calcium concentration.at rest. Clausen and Flatman (1977; Flatman and Clausen, 1979) have presented strong evidence for such a mechanism in skeletal muscle. This mechanism would predict a negative inotropic effect of ß-adrenergic stimulation after strong blockade of slow inward current, and this effect has been verified in experiments (no curves presented). More work at the biochemcal and functional levels is however needed to clarify this question. The negative inotropic effect of ß-adrenergic stimulation may in part be due to an accelerated relaxation (effect on the calcium ATPase of the SR) and the effect of cAMP-protein kinase on sodium-potassium ATPase activity is currently a subject of controversy (see for example Braughler et al., 1978).

The curve 5 in figure 8.9 is a simulation of the interaction of adenosine and an increased bath calcium concentration as these effects would logically be represented in the simulation program. The increased extracellular calcium would result in an increased intracellular calcium concentration and thereby an increased SR-fill level at rest and rate of refilling. Also, the amount of calcium entering the cell via sodium-calcium exchange from the extracellular medium is logically increased. The parameter for slow inward current from the extracellular fluid is set slightly lower than for the "control" conditions of curve 4 in the assumption that the effect of adenosine on slow inward current overrides the effect of the increased bath calcium on slow inward current. Phosphorylation parameters are left as in curve 4. As in real atria, this situation leads to a pseudo-reduction of the Bowditch part of the frequency-force relationship and to an enhancement of the Kruta part of the relationship.These effects are all ex-

plained by the effect of an increased intracellular calcium concentration on the final fill level of the SR and its rate of refilling. The shift to higher frequencies of the Kruta part of the frequency-force relationship masks the Bowditch part.(compare with curve 2C in figure 4.3).

8.3.7. Effect of an extreme acceleration of NIEA decay in atrium and the simulated system.

As described above an acceleration of the NIEA decay rate results in a shift of the Kruta part of frequency-force relationships to higher frequencies. Besides increasing the bath calcium or heart glycosides, an increase of the bath temperature also helps to accelerate the NIEA decay. Under any conditions where the Kruta part of the relationship is very strongly shifted to higher frequencies, a "bump" developes in the lowest frequency range both in model and in atrium. This is demonstrated in figure 8.10.

In the model this bump comes about because a large amount of calcium is available to the slow calcium channels from the SR at the rested state contractions. Thus, the phosphorylation of only a relatively small number of slow calcium channels in the low frequency range results in accumulation of frequency inotropy. As frequency is increased from the rested state, force first rises as a re-

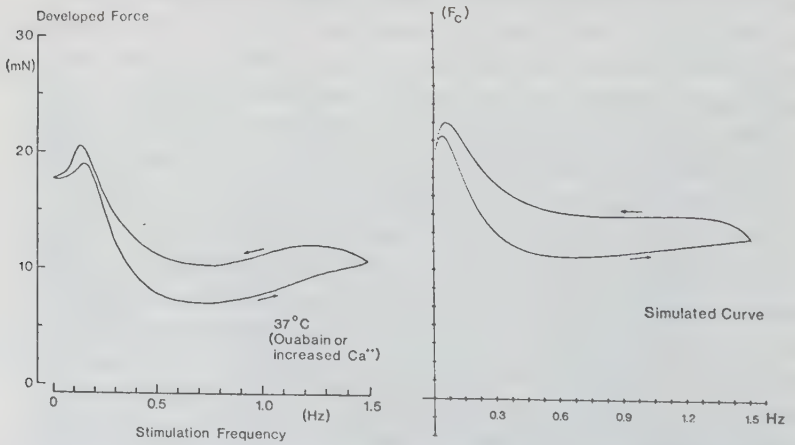


Figure 8.10 Frequency-force loops from atrium and simulated model with accelerated NIEA decay rates. See text for further explanations.

flection of the amplified PIEA I mechanism. Then, at higher frequencies the SR does not have time to refill to the rested state level and force falls with increase of frequency (the normal NIEA mechanism). At a still higher frequency range the normal Bowditch curve part is visible, although it appears more suppressed than it actually is. The mechanism of the "bump" is not normally manifest because the loss of SR-calcium in the Kruta part of the relationship completely overplays accumulation of phosphorylated slow calcium channels; occasionally NIEA decay will be quite rapid even under control conditions at 32°C (2.5 mM Ca^{++}) and the bump will occur without further manipulations.

Here is a sure-fire recipe for creating a bump in the frequency-force relationship of any guinea pig atrium: First increase the bath temperature to 37°C. Now increase the bath calcium to 7 mM. Contraction force will be close to the contractile ceiling of the preparation. Therefore, add a little carbachol, adenosine, nifedipine or verapamil until contraction force at intermediate frequencies reaches an intermediate level. The effects of the above agents and the increased bath calcium will antagonize one another on the slow inward current, but the Kruta part of the frequency force relationship will remain strongly shifted to higher frequencies. The appearance of the "bump" is guarantied. ...P.S. Don't be surprised if a second small bump also appears; the interval-strength relationship is more complex and reveals more about processes underlying contractility than commonly appreciated.

8.3.8. Effect of ouabain in atrium and simulated model.

In the left panel of figure 8.11 the typical effect of ouabain on the frequency-force relationship is presented when the rested state contractions under the control conditions are not too large. Under such conditions the positive inotropic effect of ouabain is inversely frequency-dependent; the effect is greater at the rested state and in a low frequency range than at higher frequencies. In the right panel the logical effect of an increased intracellular sodium concentration in the simulated system is presented. The rest fill level of the SR would be increased due to an increased intracellular calcium concentration,

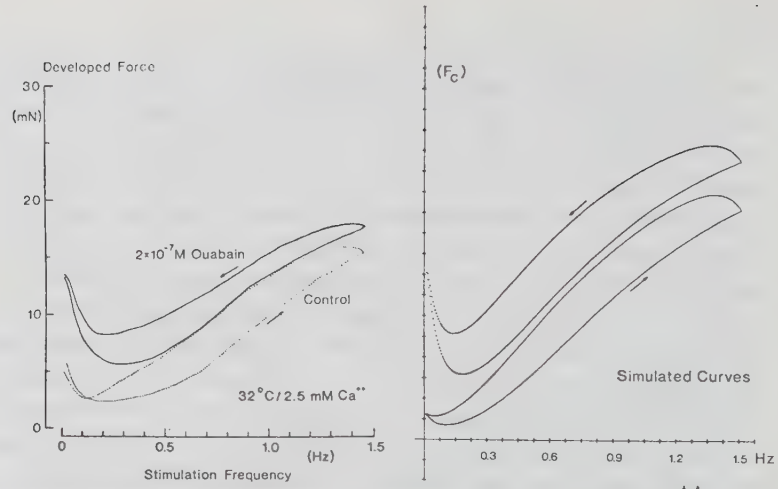


Figure 8.11 Effect of ouabain (left) at 32°C, 2.5 mM Ca^{++}. In simulated curves (right) the effect of increasing the rest fill level of the SR together with an increase of the % SR-calcium taken up by the sodium-calcium exchanger is demonstrated.

and the fraction of released SR-calcium taken up again via sodium-calcium exchange would be increased. Also, the amount of extracellular calcium entering the cell via the exchanger would be increased. The result of these changes is the typical inverse, frequency-dependent effect of ouabain. The effect on sodium-calcium exchange is basically frequency independent. The effect on the fill level of the SR at rest is greatest at the lowest frequencies, because at higher frequencies the SR does not have time to refill.

The effect of heart glycosides presented by Koch-Weser et al. (1962, 1964) on kitten atrium was somewhat different; those authors reported only a parallel upward shift of the relationship by ouabain with no preferential effect on the rested state contraction. Since rested state contractions in kitten atrium under the conditions studied come close to the contractile ceiling even without ouabain, the positive inotropic effect of ouabain on the rested state contractions in their study may well have been underestimated. Occasionally, I have had atria in which the slope of the Bowditch part of the relationship was somewhat increased by ouabain.

This may reflect an increased tendency of intracellular sodium to increase with increase of frequency after ouabain treatment. This effect is however not large enough to explain why the intercept of PIEA I decay curves is increased by ouabain (figure 7.15). In the model this effect results from the shift of the Kruta part of the frequency-force relationship to higher frequencies, so that more calcium is made available to the slow calcium channels at 0.25 Hz. In this way the PIEA I decay line may be amplified even though the Bowditch part of the frequency-force relationship does not become greatly steeper.

8.4. Addition of sodium to the simulation system.

The frequency-independent positive inotropic effect of ouabain on the Bowditch part of the frequency-force relationship raises again the question of why sodium does not seem to markedly increase intracellularly with increase of frequency in atrium. Two assumptions allow a simulation of the intracellular sodium concentration in a way to leave frequency-force loops, frequency jumps and pharmacological interventions quite realistic. First, what mechanism may work against an intracellular sodium accumulation with increase of frequency? As pointed out in 7.5 the sodium-potassium pump is electrogenic; that is, it extrudes 3 Na^+ and pumps in 2 K^+. In this way it contributes slightly to the size of the membrane potential. Due to its electrogenic nature the pump is operating both against a concentration gradient and an electrical gradient at rest. During depolarization the electrical gradient against which the pump is operating is removed, and it seems logical to assume that the pump will be stimulated at each depolarization. Second, it is assumed that the decay of PIEA IV from rest decay curves (figure 7.8) corresponds to the correct rate of sodium extrusion at rest.

A crude simulation of the intracellular sodium concentration is made in the following way on a beat-to-beat basis: The intracellular sodium concentration at rest is 1, and all calculations are related to 1 (equivalent to 7 to 10 mM free concentration). The influx per beat can be assumed to be anywhere from 20 to 100 µmol per kg wt.wt.

in accordance with the hypothetical mechanism described, a small fraction of the intracellular sodium concentration is subtracted at each beat. Assuming a quite large influx per beat (60 μ mol/ kg wt.wt.) and an intracellular sodium concentration of 9 mM at rest, about 0.2% of the free intracellular sodium or about 20 u mol/ kg wt.wt. would have to be extruded at each beat to keep the intracellular sodium concentration from accumulating by more than 10% between the rested state and 1.5 Hz. Finally, three times the amount of calcium entering the cell via sodium-calcium exchange during activation and between beats is subtracted from the intracellular sodium amount (see 8.5. for an explanation of the calculation). Between beats the intracellular sodium concentration is assumed to return toward equilibrium in a simple exponential fashion.

The intracellular sodium concentration is then coupled with the rest of the simulation system at two points: 1) The amount of calcium entering the cell via sodium-calcium exchange and the fraction of released SR-calcium entering via the exchanger are varied parallel to the intracellular sodium concentration. 2) The equilibrium being approached by the SR and the rate of approach are also varied in parallel to the intracellular sodium concentration. This coupling results from the assumption that the intracellular sodium concentration determines the intracellular calcium concentration. Although this simulation is indeed very rough, it is enough to demonstrate how the intracellular sodium concentration might be involved in certain interval-strength phenomena.

8.4.1. Simulation of a frequency jump series.

In figure 8.12 a simulated frequency jump series is presented according to the pattern described in figure 7.5. The addition of the simulated sodium system makes such a series more realistic in one respect. The strength of contraction of the first beat after rest pauses (the rest decay curve) approaches the final rested state contraction level very slowly. The first beat after even 15 min rest period is markedly larger than the rested state (30 min).

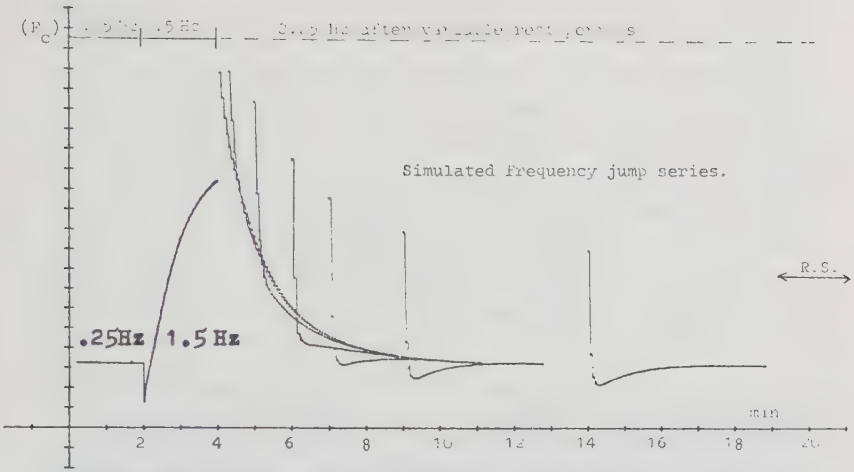


Figure 8.12. Simulated frequency jump series. Compare with the frequency jump series in figure 7.5. See text for further explanations.

This slowly-decaying process (PIEA 0) corresponds in the model to the return to equilibrium of the intracellular sodium concentration, which increases by about 7% at 1.5 Hz in comparison to the resting level. Note that the frequency jumps from 1.5 to 0.25 Hz do not show this slow phase, but rather follow the rate constant for PIEA I decay. Even if sodium would accumulate somewhat further at 1.5 Hz, the PIEA 0 would not be easily detectable in frequency jumps from 1.5 to 0.25 Hz. The SR-calcium fill level decays with dephosphorylation of slow channels in such a force transient, and the final fill level at 0.25 Hz is quite low. Since sodium-calcium exchange at activation depends strongly on the calcium released from the SR, the appearance of the PIEA 0 in a frequency jump would only be possible if the SR-fill level were quite high at the 0.25 Hz steady state. In fact, frequency jumps with increased bath calcium sometimes do show a slow decay phase which could be accountable to the PIEA 0 (no curves presented). In the rest decay curve two moments come together to make the PIEA 0 large. First, the fill level of the SR at rest changes parallel to the intracellular sodium concentration. Second, the fraction of the SR-calcium reentering the cell changes parallel to the intracellular sodium.

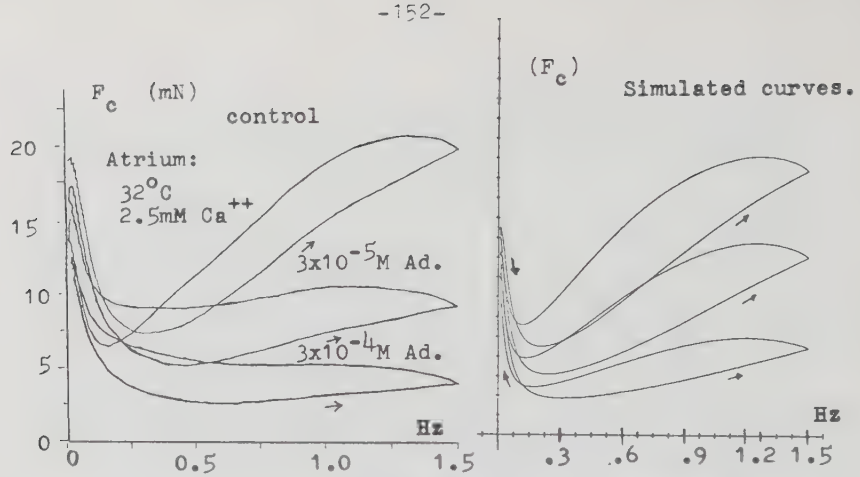


Figure 8.13. The left panel of the figure shows the representative effect of adenosine on frequency-force loops at 32°C, 2.5 mM calcium. The effect is practically indistinguishable from the negative inotropic effect of carbachol or nifedipine under these conditions. The right panel shows the effect of reducing the paramaters for slow inward current in simulated curves.

8.4.2. Simulation of the negative inotropic effect of adenosine at 32°C, 2.5 mM calcium.

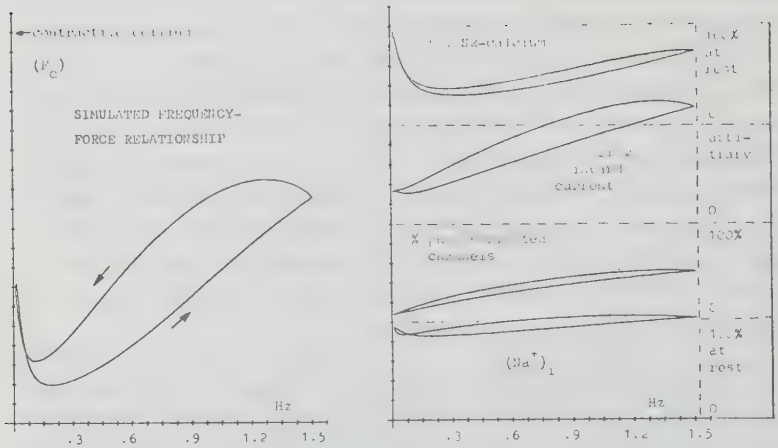
Addition of the sodium simulation to the model makes frequency-force loops for standard conditions (32°C, 2.5 mM calcium) more realistic in two respects. 1)As demonstrated in the right panel of figure 8.13, the rising and falling parts of frequency-force loops now cross in a low frequency range. This comes about in the model because quite large amounts of sodium are exchanged for calcium in the low frequency range: After each beat there is a large calcium deficit in the SR. As the SR takes up calcium from the cytosol, intracellular sodium is exchanged for extracellular calcium in relatively large amounts between beats. During a series of beats in the low frequency range (about 0.02 to 0.08 Hz) the intracellular sodium concentration would decrease. A functional correlate to this effect is seen when stimulation of atria is started after a rested state contraction at a low frequency (0.05 Hz). In the first three or four beats contraction force falls markedly (the normal NIEA), and then continues to fall more slowly for up to 30 min (no curves presented).

2) With the sodium system added to the simulation program, simulated frequency-force loops for reduction of slow inward current become slightly more realistic. With or without the sodium system rested state contractions are little affected by reducing slow inward current; the normal atrial rested state contractions are activated by sodium-calcium exchange for the most part in the model. With the sodium system frequency-force loops remain somewhat wider after reduction of slow inward current than without the sodium system. In atria as well, frequency-force loops do usually not become as narrow, as expected by simulation without the sodium system, after treatment with agents which reduce slow inward current.

To summarize, there are some interval-strength phenomena in atrium which may reflect changes of the intracellular sodium concentration. The major problem raised, however, is why sodium does not seem to accumulate markedly with increase of frequency. As demonstrated in the preceding curves, the assumption that the sodium-potassium pump is stimulated at each depolarization is a logical and adaquate way of accounting for regulation of sodium with frequency change in atrium. The way in which the intracellular sodium concentration is simulated would leave open the potential for a marked accumulation of sodium under special conditions, for example after veratridine treatment or at very high stimulation frequencies.

8.4.3. Summary of the model.

Figure 8.14 summarizes what happens in the simulated model during frequency-force loops. The total SR-calcium falls during the Kruta part of the curve and increases again during the Bowditch part. Slow inward current would decrease slightly during the Kruta part and increase during the Bowditch part. The number of phosphorylated channels would increase during the entire curve with increase of frequency. The intracellular sodium concentration would decrease in the Kruta part of the curve and increase slightly in the Bowditch part. The primary mechanism of the Kruta part is a loss of calcium from the SR; the primary mechanism of the Bowditch part is a beat-dependent phosphorylation of slow calcium channels (= "NIEA and PIKA").



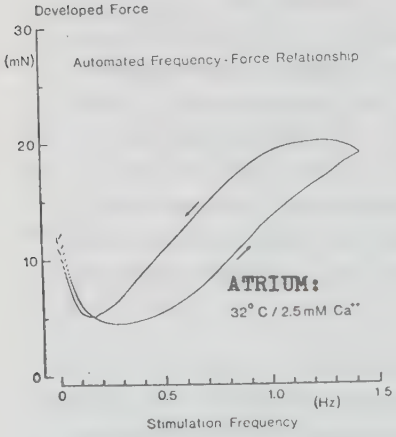


Figure 8.14. The upper right panel gives a simulated frequency-force loop with parameters set to correspond to the atrium in the lower left panel. Changes of the most important simulated entities during the frequency-force loop are given in the upper right panel.

Parameter settings for the frequency-force loop are as in figure 8.3. The parameters for simulation of intracellular sodium correspond to the description in 8.4. (see also 8.5).

8.5 Simulation program.

Store identification:

R1= frequency change/beat
R2= frequency
R3= beat interval

R80= deviation of Na_i from equilib.
R81= Nai (equilibrium = 1)
R82= fraction of Na_i pumped out/beat
R83= conversion of ^{i}Ca-in to Na-out for Na-Ca exchange
R84= rate constant of Na-pump (rest)
R85= Na-in per beat

1) R40= SR rest equilibrium
R41= R40 adjusted for Na_i
2) R42= Ca-exchange rate from SR
R43= R42 adjusted for Na_i
R44= actual SR-Ca level
R45= SR-Ca level of last beat
R47= **ΔSR-calcium from last beat**
4) R48= **releasable fraction of SR-calcium**
R49= potentially releasable Ca
3) R50= rate of diffusion in SR
R51= actual releasable Ca

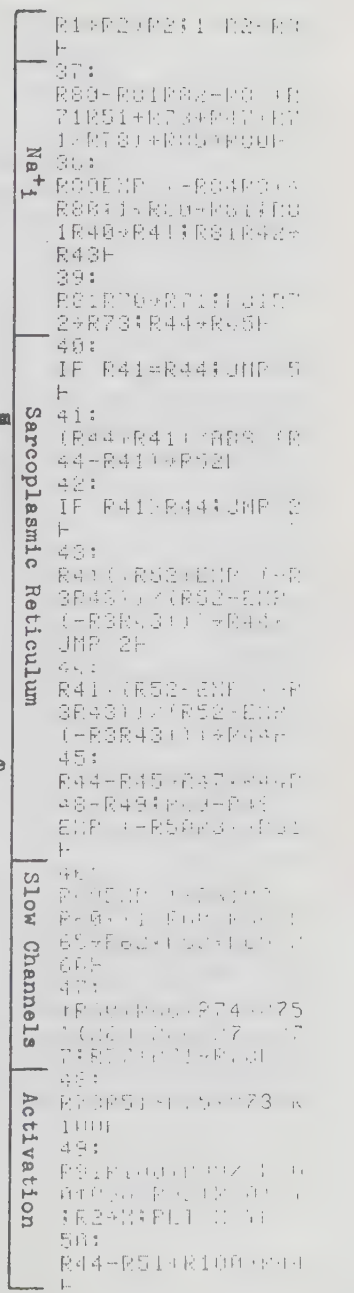
R60= fraction phosphorylated channels above rest level
11) R61= dephosphorylation rate
R62= fraction phosphorylated/beat
10) R65= fraction of non-phosphorylated channels phosphorylated/beat
9) R66= phosphorylated fraction at rest

7) R70= fraction of released SR-Ca reentering via Na-Ca exchange
R71= R70 adjusted for Na_i
5) R72= extracellular Ca-in via exchange
R73= R72 adjusted for Nai
6) R74= extracellular Ca-in via channels at 100% phosphorylation
R75= actual Ca-in via channels
8) R76= fraction of released SR-Ca in via channels at 100% phosphor.
R77= actual fraction of released SR-Ca in via channels
R78= total fraction of released SR-Ca reentering

R100= final activator Ca amount

R90= "n" of Ca-force relation
R91= max. of Ca-force relation
R92= EC_{50} of Ca-force relation

The program at right is used as a calculation loop to determine the strength of each beat. For most curves presented the sodium part of the loop was not used. **Numbers give parameter numbers in 8.2.4.**



Explanations of the simulation program.

The first program line gives exponential frequency change for frequency-force loops (see also figure 3.3).

Sodium amounts and concentrations are related to "1" as sodium concentration at rest. The conversion factor of calcium-in to sodium-out via the exchanger (R83) is calculated to be about 0.0002 for the arbitrary calcium units used in simulation: 50 arbitrary calcium units correspond to 50% force development on the calcium-force relationship. About 15 nmol/g wt wt activator calcium is thought to be needed for 50% force development (see Beresewicz and Reuter, 1977). If all of this calcium would enter via sodium-calcium exchange, about 90 umol/kg intracellular water sodium would be lost from the cell. That would be about 1% of the free intracellular sodium concentration. The conversion factor would be 0.01/50=0.0002. This factor was used in all simulations presented with the sodium part of the program. Even with less favorable calculations the intracellular sodium concentration would decrease somewhat in the Kruta part of the frequency-force relationship under the assumption that the calcium for refilling of the SR between beats is supplied by sodium-calcium exchange at the sarcolemma. It is assumed in the present form of the program that part of calcium exchange to the cytosol from the extracellular fluid at rest takes place via "leaky" slow calcium channels. If this assumption is left out the fall of intracellular sodium in the Kruta part of the relationship would be slightly greater.

Lines 40 to 44 give the equation system derived for calcium exchange between cytosol and SR at rest. Line 45 determines the amount of releasable calcium, according to the assumption that calcium at release sites will become available from up-take sites exponentially. An inaccuracy results in that calcium not released must rediffuse to release sites between beats in the program's present form. With the discrete simulation method it is hard to overcome this inaccuracy; it would be necessary to calculate the calcium gradient through the SR after a beat, and this gradient would be important in determining at what points along the SR more or less calcium would be taken up at the next beat.

The rest of the program should be understandable by following the program with the store identification list.

The interested reader will have little difficulty in finding gross oversimplifications in the simulation program. The inaccuracies which result by the discrete simulation method cannot, however, account for any of the points where simulated curves and the behavior of real atria coincide. A more sophisticated simulation can be expected to result in more points of agreement between simulated model and atrium, and not less.

8.6. Summary of chapter 8.

1) Two hypotheses allow a rough, digital simulation of the atrial interval-strength relationship:
   i. Calcium is released from the terminal cisternae of the sarcoplasmic reticulum to the glycocalyx with each depolarization.
   ii. A small fraction of unphosphorylated slow calcium channels is phosphorylated at each beat.

2) The simulated system describes well:
   i. Frequency-force loops obtained by the automated method used in this dissertation at 32° and 28°C.
   ii. Recovery of contractility after rested state contractions under various conditions.
   iii. The frequency-dependence of force transient form for reduction of frequency in a step.
   iv. The contractile response to extrasystolic beats.
   v. The interactions of adenosine and nifedipine with orciprenaline on the frequency-force relationship.
   vi. The form of frequency-force loops under conditions where the "NIEA-decay rate" is accelerated.
   vii. The effect of ouabain on the atrial frequency-force relationship.
   viii. The behavior of force transients in frequency jump series.
   ix. The negative inotropic effects of nifedipine, carbachol and adenosine on the normal frequency-force relationship, and the interactions of these substances with increased bath calcium.

9. Final discussion.

The results presented in chapters 4, 5, 6, and 7 were discussed at the end of those sections. Chapter 8 essentially presented an interpretation of those results. Therefore, what is left to be discussed is the interpretation of results. It was already pointed out that attempts to fit findings to previous models of electromechanical coupling to date were not successful. This is of course no proof that the proposed model is correct, and it is certainly possible that some other model will be conceivable to someone else and account for the functional findings. If that is the case, only harder evidence and a critical consideration of the necessary assumptions will help to distinguish which of the models is more likely to be correct. The simulated model was an important way of testing whether the proposed model of excitation-contraction coupling could deal with the phenomena described. It is not true that any set of 8 or 10 variables can be strung together in a simulated system to produce the interval-strength relationship of atrial muscle and its response to a large number of pharmaca. The current array of ideas being discussed to explain excitation-contraction coupling in heart could perhaps be somewhat reduced, if each of these concepts were put to the test of a simulation. It is hoped that the present simulation will be accepted as a demonstration that the model at question deserves serious further consideration.

Previous simulated models have accepted the most widely-held opinions about how contraction activation takes place and can be regarded as attempts to demonstrate that those concepts are adequate. Although the present model accepts many individual aspects of previous models, the movement of the arrow from the terminal cisternae to the cytosol at activation, to the glycocalyx at activation is a major change. And the movement of this arrow anticipates fairly major developments in this field in the next years. The mechanism of calcium release from the sarcoplasmic reticulum would bring a number of advantages for the heart

cell: The gate mechanism in the couplings of the terminal cisternae would be simpler than either a calcium-induced or depolarization-induced release of calcium via pores in the terminal cisternae to the cytosol. Second, the strict routing of calcium through the SR and back to the glycocalyx would provide a very rapid, efficient way of regulating the amount of activation. The primary control would be kept at the sarcolemma, and would respond immediately to changes at the sarcolemma. Third, the movement of calcium to the SR, then to the glycocalyx, and back into the cell via sodium-calcium exchange would be economical. The energy used to move calcium from the cytosol into the SR would be partially regained by extruding sodium from the cell. Finally, the identity of the mechanism of calcium release from the SR and the mechanism of calcium extrusion from the cell would provide a very effective safeguard against calcium "over-loading" at high frequencies or with catecholamines.

The limitations and inadequacies of the simulated model should not be overlooked. First, three effects of frequency change were ignored. The PIEA II has no couterpart in simulated curves. If this phase of frequency inotropy decay would correspond to the role of mitochondrial calcium uptake and release in the interval-strength relationship, then a more sophisticated simulation would be necessary to properly describe this process. A slow negative effect of activation was also ignored; this effect is seen in frequency jumps with β-adrenergic stimulation antagonized by various negative inotropic agents. Although it is only visible under such conditions, it likely is present under normal conditions as well. Also, the effect of metabolic fatigue is ignored, although it probably plays a part in the form of many frequency-force loops. Second, the restitution of contractility between beats is unrealistic in that contraction force does not decline toward zero at the smallest intervals (see figure 8.6), as is the case in real atria. Also, the apparent acceleration of restitution with increase of frequency or other positive inotropic interventions (Kruta, 1964) is not duplicated in

the simulated model. Finally, the exact behavior of the PIEA III is not duplicated after extrasystolic beats at different frequencies (figure 7.3); that is, the "overly" beat-dependent nature of the PIEA III (pp. 98-99) is not adequately described by the simulation system.

There is good reason to think that the above inadequacies reflect only the limitations of the simulation program and not the electromechanical coupling model. Particularly the representation of the sarcoplasmic reticulum and the glycocalyx in the simulated model are such gross simplifications that inadequacies must be expected. Much more, it seems noteworthy that such a simple digital simulation could deal with so many phenomena. What is now needed at this level is a more detailed simulation, which would be able to deal with the more rapid processes controlling contractility. Such a simulation should be able to produce an action potential and contraction analogue. Most importantly, it should be able to deal with the expected behavior of the glycocalyx as diffusion barrier in detail. If the present excitation-contraction coupling model is correct, the glycocalyx will be of major importance in regulating the ionic composition at the surface of the unit membrane, particularly for calcium. Such a simulation should be able to represent diffusion of calcium from the extracellular fluid to the unit membrane, lateral diffusion from the couplings along the unit membrane, and diffusion away from the SR couplings.

Up to now the idea that calcium might enter the cell at each beat via sodium-calcium exchange in mammalian heart has been proposed primarily by the group of Langer and coworkers. One of the main reasons why this proposal has not received a larger support is that sodium-calcium exchange has been considered to be electroneutral, and for such a system there would be no clear reason for exchange of calcium in the inward direction to be favored at depolarization. Even with new direct evidence for a

3 Na^{+} / 1 Ca^{++} exchange ratio in heart (Pitts, 1979; Caroni and Carafoli, 1980) the projected intracellular calcium concentration at action potential overshoot and at equilibrium for sodium-calcium exchange would only be in the range of 10^{-6}M (see 8.1), hardly large enough to anticipate a considerable calcium influx via the exchanger. If however, the calcium concentration in the glycocalyx near to SR-sarolemma couplings reaches values greater than in the extracellular fluid at activation, there indeed would be reason for a large calcium influx via sodium-calcium exchange in those areas. One functional arguement against such an exchange can be countered by this order of things: Blockade of slow calcium channels in ventricle leads to complete or nearly-complete loss of contractility. Where and how should sodium-calcium exchange into the cell with each beat take place? According to the present model the calcium entering with each beat via sodium-calcium exchange is largely of sarcoplasmic origin, and indirectly it entered the cell at previous beats via slow calcium channels. The only real example of contractility in the absense of a slow inward current or with very little slow inward current would be the normal rested state contractions of atrium. Even after slow channel blockade they remain strong, because the SR of atrium fills up to a quite high level at rest. It would be interesting to know whether the large rested state contractions, which can be produced in guinea pig papillary muscle in magnesium-free solution, (Vierling and Reiter, 1975) are also insensitive to nifedipine.

The next years will show whether or not the hypotheses presented in this dissertation are correct. At the functional level there remains lots to be done. In particular, it will be important to examine to what extent interval-strength phenomena in mammalian ventricle correspond to the patterns found in atrium. In papillary muscle contraction force <u>rises</u> in a strongly beat-dependent phase upon stimulation after long rest periods (Seibel, 1975). This phase seems to be just the flip-side of the situation in atrium, where force <u>falls</u> in a strongly beat-dependent phase upon stimulation after long rest periods. It is tempting

to equate these two phases. In ventricle the amount of calcium taken up by the SR from one beat to the next in this phase would be greater than the amount lost, whereas in atrium just the opposite seems to be the case. If the beat-dependent phases in atrium and ventricle are indeed related, the beat-dependent phase in ventricle should also be predictably influenced by agents which would change either the fraction of SR calcium taken up via sodium-calcium exchange or slow calcium channels. Seibel (1975) reported no such changes; it would be important to re-examine this question using frequency jump series as presented in this dissertation*. Although a sodium-dependent phase of frequency inotropy production clearly dominates in guinea pig papillary muscle (Seibel, 1975), it seems unlikely that the cAMP-dependent phase (PIEA 1) of guinea pig atrium is completely lacking. To complete the range of variation, it seems that kitten papillary muscle shows neither a predominant sodium-dependent phase or cAMP-dependent phase. According to relationships presented by Kaufmann et al. (1974) very little frequency inotropy accumulates in that preparation above 6 beats/min, a very flat frequency-force relationship in comparison to either guinea-pig atrium or papillary muscle. The accumulation of frequency inotropy in kitten papillary muscle might indeed correspond to a relatively pure accumulation of calcium in the SR, a negative picture of the Kruta part of the atrial frequency-force relationship. It should be possible in the future to systematically classify the various interval-strength phenomena found in various preparations in light of a better understanding of how they might come about and a more detailed description of their kinetic and pharmacological behavior.

Although the hypothetical model of excitation-contraction coupling presented in chapter 8 seems to be able to account excellently for the functional and pharmacological behavior of the atrial interval-strength relationship, more direct evidence would certainly be preferable. At the electrophysiological level it should be possible to

*Note added in final proof: A clear slowing of the beat-dependent phase (PIEA III) with increased accumulation of frequency inotropy has since been verified for guinea pig papillary muscle.

find an electrophysiological correlate to an increased slow inward current with increase of frequency. The maximal velocity of depolarization under slow response conditions in guinea pig atrium has a clear tendency to decrease with increase of frequency (Thyrum, 1974), perhaps a reflection of an increased outward potassium current with increase of frequency. It might be possible to separate this effect from an increased slow inward current by examining slow responses during an entire force transient for increase or decrease of frequency.

It will be more difficult to prove or disprove the proposed mechanism of calcium release from the terminal cisternae of the SR. The atrial rested state contractions could provide a small bit of leverage. If the electromechanical coupling model is correct, then 3 or 4 times as much calcium needed for activation of a single beat is lost from the cell during a rested state contraction in atrium. It should be possible to pick up such a large calcium extrusion as a small peak in Ca^{45}-exchange experiments. That would at least be an indication that calcium efflux, as well as calcium influx, is beat-dependent. A more direct proof of the release mechanism might be possible with calcium indicators. Related to the extracellular space, the calcium released at the rested state would raise the extracellular calcium concentration by somewhat more than 1%. An extracellular calcium signal before onset of contraction would be the most dramatic experimental result which the present model predicts, and time will tell whether such a signal is found.

10. Summary of the dissertation.

1) An automated method for registration of frequency-force relationships in isolated heart muscle preparations was used to study the frequency-force relationship of guinea pig atrium under a wide range of conditions.

2) Blinks' and Koch-Weser's analysis of the production of the interval-strength relationship in atrial muscle was expanded and applied to a number of pharmacological interventions. Special behaviors and relationships of the "inotropic effects of activation" were discovered.

3) Functional findings are difficult or impossible to explain in terms of the hypotheses proposed to date to account for the production of frequency inotropy in mammalian atrium. Findings are accounted for well by the hypothesis that phosphorylation of slow calcium channels by the catalytic subunit of cAMP-dependent protein kinase is facilitated by depolarization.

4) Biochemical work helps to eliminate other possible cAMP-dependent mechanisms of frequency inotropy production. Also, biochemical work eliminates the possibility that the negative inotropic effect of adenosine in guinea pig atrium could be due to a reduction of the cAMP-level.

5) A new model of excitation-contraction coupling in heart, developed by Mensing in this institute, allows a simulation of the atrial interval-strength relationship as well as the actions of many pharmacological agents on the relationship. This model assumes that the physiological mechanism of calcium release from the sarcoplasmic reticulum in heart is not a direct release to the cytosol, but rather to the glycocalyx, from where calcium would reenter the cell via slow calcium channels and sodium-calcium exchange with each beat.

6) The discrete, digital simulation reproduces accurately the normal frequency-force relationship, force transients for step frequency change, effects of extrasystolic beats; effects of ß-adrenergic stimulation, increased bath calcium, heart glycosides, adenosine, carbachol, nifedipine, and the bath temperature on the frequency-force relationship are also simulated well.

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Lebenslauf

Am 20. 8. 1952 wurde ich, Donald William Hilgemann, als Sohn des Ernest William Hilgemann (deutscher Abstammung) und seiner Ehefrau Pricilla, geb. Nelson (norwegischer Abstammung), in Postville, Iowa, U.S.A. geboren. Die Schule besuchte ich von 1957 bis 1970 in den Bundesstaaten Iowa und Nebraska mit Abschluß an der Valley High School in Elgin, Iowa. Im Juni 1970 begann ich ein Studium an der University of Iowa, Iowa City, in den Fächern Biologie, Literaturwissenschaft, Fotographie und Filmproduktion. Im Februar 1972 unternahm ich eine Reise nach Europa, wobei ich nach verschiedenen Jobs ab Wintersemester 1973 Biologie studierte an der Eberhard-Karls-Universität in Tübingen. Dort absolvierte ich im Juli 1974 die Prüfung zum Vordiplom. Im Wintersemester 1975 legte ich die theoretische Hauptdiplomprüfung im Hauptfach Zoologie und in den Nebenfächern Pharmakologie, Biochemie und Humanphysiologie ab. Die Diplomarbeit wurde von März 1976 bis Janaur 1977 am Pharmakologischen Institut, Tübingen, unter Anleitung von Prof. Dr. M. Siess durchgeführt. Am gleichen Institut arbeitete ich weiterhin von Januar 1977 bis Juni 1980 an vorliegender Dissertation. Im Sommersemester 1976 fing ich ein Zweitstudium in Humanmedizin an und bestand Sommer 1977 die ärztliche Vorprüfung (Physikum).

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